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Clustering of inhibitory killer cell Ig-like receptors

By

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled “Clustering of inhibitory killer cell Ig-like receptors” submitted by Peter Daniel Borszcz in partial fulfillment of the requirements for the degree of Master of Science

For Laura

Abstract

Natural killer (NK) cells are inhibited by killer cell immunoglobulin-like receptors (KIR) that bind major histocompatability complex (MHC) class I ligands. Recent literature has shown that NK inhibitory receptors, including KIR, cluster at the interface with ligand bearing target cells. Receptor clustering is known to be required for signal generation of other receptors that transmit activation and proliferative signals. The role of receptor clustering for inhibitory signal generation has not been described.

In this thesis, we show that a KIR receptor variant with EGFP at the N-terminus fails to form macromolecular clusters however continues to generate an inhibitory signal. Additionally we show that this receptor, at low receptor levels, is unable to signal to the same degree as a KIR receptor with EGFP at the C-terminus. These data suggest that KIR clustering is not required for inhibitory signalling but may increase inhibitory signalling.

In addition to the consequences of KIR clustering we also investigated the prerequisites of KIR clustering. We found that KIR clustering is dependant upon an active actin cytoskeleton. We further investigated the interrelationship between KIR and the cytoskeleton and found that KIR clustering excluded areas of increased actin polymerization. These results suggest that KIRs, by regulating actin cap formation at the interface, are responsible for controlling the formation of the activating immunological synapse.

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List of Abbreviaions

Ab - Antibody

ADCC – Antibody Dependent Cell Cytotoxicity

APC – Antigen Presenting Cell

BATDA – bis (acetoxymethyl) 2,2':6',2"-terpyridine- 6,6"- dicarboxylate

CD – Cluster Designation (ie; CD156a)

CTL – Cytotoxic T Lymphocyte

EBV – Epstein Barr Virus

EGFP – Enhanced Green Fluorescent Protein

EGFR – Epidermal Growth Factor Receptor

FACS – Fluorescence Activated Cell Sorting

FCS – Fetal Calf Serum

GM-CSF – Granulocyte Macrophage Colony Stimulating Factor

HCMV – Human Cytomegalovirus

HSV – Herpes Simplex Virus

HIV – Human Immunodeficiency Virus

HLA – Human Leukocyte Antigen

ICAM – Intercellular Adhesion Molecule

INF - Interferon

ITAM – immunreceptor tyrosine activation motif

ITIM – immunoreceptor tyrosine inhibitory motif

KHSV – Karposi's Sarcoma Herpes Virus

KIR – Killer Cell Ig-like Receptor

LAT – Linker for Activated T cells

LFA – Lymphocyte Function associated Antigen

MCMV – Mouse Cytomegalovirus

MHC – Major Histocompatability Complex

MIP – Macrophage Inflammatory Protein

MTOC – Microtubule Organization Center

NK – Natural Killer

PI3K – Phosphatidylinositol 3-kinase

PKC – Protein Kinase C

PLC – Phospholipase C

SLP – SH2 domain containing Leukocyte Protein

SH – Src Homology

SHP – SH2 domain containing phosphatase

SMAC – Supramolecular Activation Custer

TCR – T Cell Receptor

TNF – Tumor Necrosis Factor

ZAP – Zeta Associated Protein

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Chapter 1. Overview of NK cell function

1.1 Natural Killer Cells

Natural Killer (NK) cells are cytolytic lymphocytes that target cells infected by intracellular pathogens (1-3). Historically, NK cells are classified as large granular lymphocytes similar to cytotoxic T cells (4, 5). Populations of NK cells are defined by the expression of NK cell markers (ie; NK1.1) and the coordinate lack of T cell receptor (TCR) expression (6). T cells become activated by a somatically rearranged clonal antigen receptor in concert with the CD3 complex (7). In contrast, NK cells are activated for cytolysis by many invariant receptors and recognize target cells by a loss of "self" ligand expression, namely the disregulation of Major Histocompatibility Complex (MHC) class I expression by viruses and other intracellular pathogens (Figure 1) (8, 9).

Upon contact with an infected cell, NK cells reorient their microtubule organization centre (MTOC), facilitating granule exocytosis toward the target cell (10, 11). NK cells release granules that induce apoptosis, or programmed cell death, in the target cell thus eliminating the infected cell and preventing further infection (12). In addition to mediating the lysis of infected targets NK cells also release cytokines with immunoregulatory activities. These include the pro-inflammatory cytokines INF-γ and TNF, as well as the proliferative cytokine GM-CSF, and chemokines MIP-1α, MIP-1β, and RANTES (13). NK cytotoxicity, in combination with the potential to modulate the immune response by releasing soluble factors, makes these cells well suited to function during the initial phases of the immune response.

Figure 1. *NK Cell Target Recognition*. NK cell recognize their targets by the altered expression of MHC class I via inhibitory receptors (KIR) expressed on their cell surface.

During an immune response, NK cells provide a mechanism to combat viruses that have evaded cytotoxic T cell (CTL) responses, and thus have often been viewed as a companion lymphocyte to T cells. CTLs generate an immune response after becoming activated in response to intracellular antigen-derived peptides that are presented on MHC class I molecules. CTL utilize an antigen specific TCR to probe potential targets for MHC/nominal antigen expression. Once the TCR has been engaged, CTL are effective at destroying cells that are infected with viruses and other intracellular pathogens. The CTL can initiate apoptosis of infected target cells by releasing cytotoxic granules or by engagement of Fas ligand (14). Apoptosis of infected cells destroys the ability of these cells to harbour and propagate virus, thus attenuating viral infection. However, some viruses have adapted to evade the T cell immune response by downregulating MHC class I expression, thus preventing recognition of the infected cell by antigen specific TCR (15-17). NK cells are thought to be adapted to respond to these viruses where T cell immunity is insufficient to contain an infection.

1.11 Role of NK cells in the immune response

The importance of NK cells in early defense has been demonstrated in mouse models utilizing NK depletion experiments. These experiments show an increased sensitivity of mice to a range of viral infections including mouse cytomegalovirus (18), herpes simplex virus (19), influenza (20), and Coxsackie virus (21). Mice challenged with these pathogens following NK depletion

experience higher morbidity and mortality due to the progression of the infection. In addition, transgenic mice with a selective NK cell deficiency exhibited reduced capacity to clear targets that failed to express self MHC (22).

The findings in mice are mirrored by epidemiological data in humans. Low NK cell cytolytic activity in humans is associated with increased sensitivity to herpes simplex virus (HSV) (23), Epstein-Barr virus (EBV) (24), and human cytomegalovirus (HCMV) (25). Direct evidence of the importance of NK immune function in humans is exemplified by the case of a female patient in which no cells displaying NK cell markers or lymphocytes exhibiting NK cytotoxicity were observed (23). The patient's acquired immune responses, such as antibody titer and memory T cell responses appeared normal. She first presented with an overwhelming chicken pox infection. After overcoming this initial infection she subsequently developed life threatening HCMV and HSV infections. In patients with a normal immune system these infections are rarely life threatening, either being quickly cleared or controlled to an asymptomatic level. Cumulatively, these data indicate a vital role for NK cells in the immune response to viral infections and suggest an important role for response initiation, especially so for those infections with large DNA viruses (ie; HCMV, HSV, and EBV).

The central role of NK cells in early control of viral infection makes this branch of the immune system an ideal target for viral immune evasion strategies. Unlike its T cell counterpart, NK cells have developed a multiple receptor system with numerous activating and inhibitory components that work together to distinguish healthy "self" from infected "nonself" target cells. The complex

interactions of multiple inhibitory receptors are thought to make viral evasion of NK cytolysis difficult; there are few viruses that can coordinate the inhibition of expression of MHC, to evade CTLs, without becoming susceptible to NK cytolysis. However some pathogens, for example HIV, selectively downregulate only some MHC isotypes, putatively to avoid both NK and T cell immune responses (26). Using an alternative strategy, MCMV encodes a MHC class I homologue, UL18, which prevents NK activation meanwhile being expressed in place of nominal antigen/ MHC capable of activating T cells. MCMV mutants with a UL18 gene deletion suffer a significant reduction in viral propagation due to NK control of virus replication *in vivo* (27). Karposi's sarcoma herpes virus (KHSV) is an example of another virus that has developed a mechanism for avoiding the NK cell immune response. KHSV K5 protein downregulates expression of both ICAM-1 and B7-2, NK adhesion and activation receptor ligands, on infected cells to prevent the activation of cytolysis pathways (28). That viral pathogens attempt to escape NK cell lysis suggests an important role for these lymphocytes in the normal control of intracellular pathogens.

1.12 The Missing Self Hypothesis

Natural killer cells, unlike T and B cells, lack an antigen specific clonal receptor. A major question in the field has been: what factors allow NK cells to distinguish healthly "self" targets from those that have become malignant or virus-infected. Early literature on NK cells was confounding as to the potential mode by which NK cells recognize targets. Two different systems, *in vivo* hybrid resistance

and *in vitro* cytotoxicity, produced two different findings. Hybrid resistance, wherein an AxB mouse rejects a parental transplant, was thought to be controlled by MHC-linked genes (29, 30). In contrast, *in vitro* cytotoxicity studies showed no target cell MHC specificity or restriction (31). Moreover, some NK targets did not express any detectable levels of MHC gene expression.

A cohesive theory of NK target recognition was first described by the missing self-hypothesis (Figure 1). Proposed by Karre and colleagues it states that NK cells recognize targets through a lack of MHC class I expression (32). The missing self-hypothesis is dependent upon a constitutive activation signal between potential target cells and a perusing NK cell, which has yet to be adequately described. Recognition of "self" or uninfected target cells is mediated by inhibitory receptor interactions with MHC class I. NK cells, and some subsets of T cells, express inhibitory receptors for MHC class I that mediate this inhibition. The missing self hypothesis predicts that inhibitory interactions would dominate over the constitutive interactions that activate cytolysis, thereby preventing autoimmunity. Despite the initial simplicity of the missing self hypothesis, a large body of research shows that the receptors responsible for NK cell recognition of targets are comprised of two distinct and highly diversified families.

1.2 Human NK cell Receptors

The first NK cell receptors were originally described as inhibitory members of what have become two large diversified receptor families that recognize MHC class I (Figure 2) (33, 34). In terms of evolution, NK cell receptors have diverged

quite rapidly likely aided by the polymorphic nature of MHC class I and pathogen driven evolutionary forces (35, 36). The predominant inhibitory receptors in human and higher primate NK cells are the Killer Cell Ig-like receptors (KIR). These type 1 transmembrane receptors belong to the immunoglobulin superfamily and mediate both activating and inhibitory functions. (37). In contrast a functionally similar and structurally distinct family, the Ly49 receptors, are expressed in rodents and also been detected in baboon NK cells (36, 38-40). Despite the Ly49 receptor family being type II transmembrane C-type lectin receptors, they have many of the same signalling functions as KIR during the NK immune response (41, 42).

1.21 CD94/NKG2

A primordial branch of the C-type lectin family, namely the CD94/NKG2 complex, is shared by both murine and human immune systems. The CD94 receptor is the most highly conserved NK cell receptor between mice and humans and recognises unique MHC ligands performing identical function in both species (43). The ligand for CD94 is HLA-E in humans and Qa-1 in mice (44-47). These nonclassical MHC require the leader peptides of some classical MHC molecules (HLA-A, B, and C, or, H-2D, K and L) that bind to the HLA-E/Qa-1 peptide binding groove, stabilize the MHC, and allow expression at the cell surface (47, 48). Thus these nonclassical MHC behave as survey molecules for classical MHC translation inside the cell; where there is no classical MHC

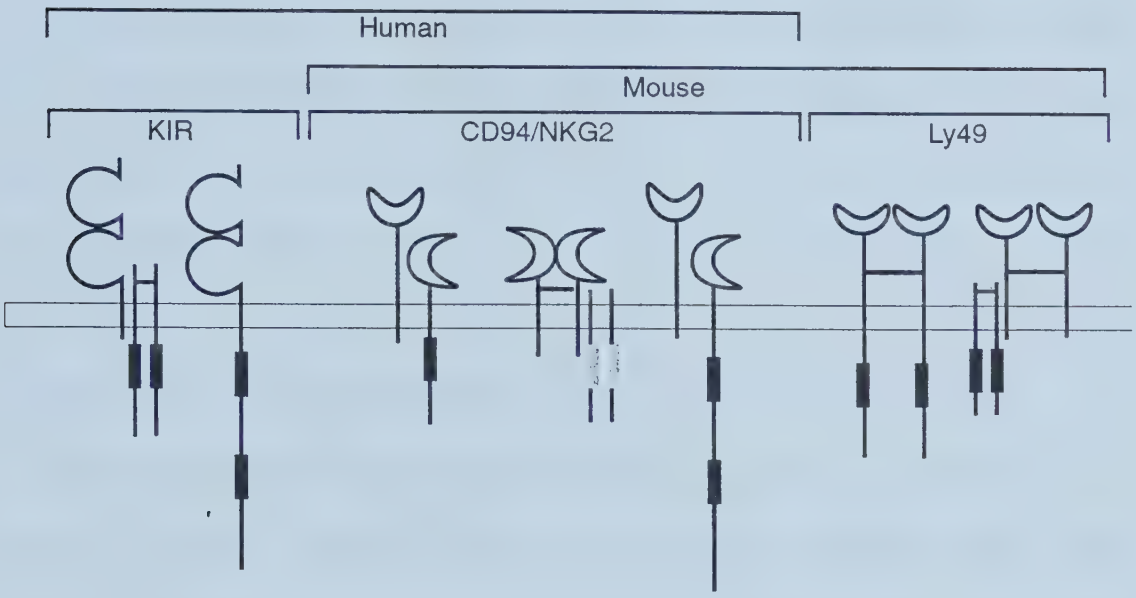


Figure 2. *NK Cell Receptors.* NK Cell receptors are composed of two distinct families; the C-type lectins, represented by CD94/NKG2 and Ly49 and the immunoglobulin superfamily represented by KIR. Human NK cells express KIR while mouse NK cells express Ly49. Both species express CD94/NKG2 receptors. The boxes in the cytoplasmic domains represent the ITAM (green), ITIM (red), and PI3K (yellow) consensus motifs.

translation there is no HLA-E or Qa-1 cell surface expression either. CD94 functions as a heterodimer with members of the NKG2 family: CD94 contacts ligand while NKG2 transduces signals upon ligation of the receptor (49). NKG2A is an inhibitory member of the NKG2 family, which functions like the inhibitory KIR receptors to inhibit NK cytolytic functions (50, 51). In contrast to NKG2A, NKG2C is an activating member of this family and functions to induce killing (52). In summary, the CD94/NKG2 receptor system in both primates and rodents acts to monitor the translation of classical MHC class I expression via the expression of the nonclassical HLA-E and Qa-1.

1.22 Killer Cell Ig-like Receptors

Killer Cell Ig-like Receptors were first described as a group of human inhibitory receptors expressed on the surface of both a subset of T cells and NK cells (37). These type I receptors are expressed as 58 or 70 kDa monomers with two or three extracellular Ig-domains, respectively. The structure of the Ig domains places these receptors within the hematopoietic receptor family (53, 54). The MHC binding site is formed between two Ig domains with the membrane proximal face of the first and the membrane distal face of the second contributing to the interaction (53, 55).

Members of the hematopoietic receptor family are known to dimerize upon ligand binding. The biochemical and crystallographic data on KIR-MHC interactions has been conflicting (56). Utilizing cobalt or zinc various groups have shown that KIR may form dimers independent of MHC ligation (57, 58). Fan *et al.*

suggested a 2:1 stoichiometry between KIR-MHC complexes, but the authors did not speculate on the number of homotypic or heterotypic interactions that may be occurring (57). The first crystal structure of a KIR engaged with ligand was KIR2DL2 bound to HLA-Cw3 (59). This structure showed that KIR might form two independent contact sites with a single HLA-C. Moreover, this crystal structure predicted that KIR multimerization, and therefore clustering, may be independent of any KIR-KIR contacts. A second crystal structure revealed more possible KIR interactions with HLA-C (55). This structure, despite a low 6 Å resolution, revealed a dimer-dimer interaction between two KIR2DL1 receptors and HLA-Cw4. This crystal predicted interactions not only between KIR and single MHC but also interactions between a KIR receptor and the MHC of the neighbouring complex. This type of interaction may play a role in promoting clustering of the receptor at the target interface. A similar mechanism of lattice formation is thought to be responsible for the promotion of clustering of CTLA-4 by B7 at the interface of T cells with APC (60).

1.3 NK cell activation receptors

NK cell membrane proximal activation signals are prerequisite to the inhibition of cytolysis, putatively to phosphorylate the ITIM and activate SHP-1 (61-63). Therefore, to study the mechanisms of inhibitory signalling requires an understanding of activation signals. One of the major differences between our understanding of the T and NK cell systems is the degree to which these activation systems have been defined. The T cell uses a TCR to recognize MHC

loaded with appropriate antigen. In addition, coreceptor and costimulatory roles for accessory receptors in T cells have been well defined. In contrast, NK cell killing is initiated by activation receptors whose ligands are unknown, thus absolute discernment of receptor function (i.e.; activation, costimulation, or coreceptor) has been difficult. For example, many of the studies that have characterized NK activation receptors have used antibody as an artificial ligand in reverse antibody dependant cell cytotoxicity (ADCC) assay to test receptor function. A reverse ADCC involves the use of an Fc receptor positive target cell and an antibody to the putative activation receptor. The effector cell activation receptor is engaged by the Fc receptor bound antibody and cytolysis proceeds. One caveat of this assay is that higher affinity interactions due to antibody ligation may enhance an *in vivo* costimulatory signal, a signal which normally augments an activating signal, to one that directly mediates effector function. In addition, many different isoforms of KIR and Ly49 activating and inhibitory receptors recognise similar ligands and these types of experiments mask potential interplay between multiple receptors (64). Using reverse ADCC and other methods, recent literature has seen a plethora of descriptions of putative NK cell activation receptors (65).

1.31 Multisubunit activation receptor complexes

An emerging paradigm in the field of hematopoeitic activation receptors is the formation of multisubunit signalling complexes (66). Key components of these complexes are small adapter proteins (ie; CD3) that bind to ligand-recognition

receptors (ie; TCR). NK and T cell adapter proteins have two key features that are necessary for function. One feature is a charged residue in the transmembrane region that is responsible for the association with ligand-recognition receptors. A second key feature of these adapter proteins is an immunoreceptor tyrosine activation motif (ITAM) in the cytoplasmic domain with a consensus sequence of $YxxL_{6-8x}YxxL/I$ (67). The tyrosine residues of the ITAM become phosphorylated upon receptor engagement resulting in the recruitment of tyrosine kinases that initiate signalling cascades (68). The archetypical activation receptor that signals in this manner is the TCR in concert with the CD3 complex. In a mechanism similar to the TCR, activating KIR receptors bind to adapter components via a charged cytoplasmic residue. The best-characterized adapter proteins in NK cells are the FcεRIγ and DAP homodimers.

DAP-12 binds to the KIR short tailed isoforms and activates src and syk family kinases through phosphorylation of its ITAM motif (69). A related adapter protein, DAP-10, binds to the NKG2D receptor and activates pathways mediated by phosphatidylinositol-3-kinase (PI3K) via a YINM motif (70, 71). The contribution of the PI3K signalling cascade to NK cytolysis remains undefined. However, CD28, a T cell costimulatory receptor that activates PI3K through an YxxM motif, can function as a receptor to activate cytolysis in YTS, an NK-like cell line, thus suggesting an important role for PI3K in NK activation and cytolysis (72, 73). Moreover, one of the hallmark steps during cytolysis is the polarization of NK cell granules toward the target. PI3K has been reported to contribute to granule polarization in NK cells (74). Whether the PI3K pathway in NK cells initiates

cytolysis, or alternatively functions in a purely costimulatory role, similar to the T cell system, remains to be determined.

On the other hand, the FcεRIγ adapter protein binds and signals for another set of NK activation receptors; CD16, and the NKpR "natural" cytotoxicity activation receptors (65). CD16 functions to activate NK cell cytolysis though antibody dependant cell cytotoxicity (ADCC) initiated when antigen is bound by CD16 associated antibody (75). In contrast, "natural" cytotoxicity utilizes receptors expressed by NK cells to recognize poorly defined ligands on target cells. Receptors responsible for the activation of natural cytotoxicity include NKp32, NKp44, NKp46, and NKp80(76-79). One ligand for NKp46 was reported to be haemagglutinin proteins of influenza, vesicular stomatitis virus, and sendai virus (80). However, it is likely that alternative ligands exist as previous work has shown functional activation through NKp46 in uninfected tumor cell lines (81). Stimulatory ligands for the remaining NKpR activating receptors have yet to be described. In review, a diverse array of NK cell activation receptors function through adapter proteins to initiate downstream signalling events including the activation of cytolytic pathways.

1.32 Role of LFA-1 integrin in activation of cytolysis

In T cells a strong linkage between activation and adhesion receptors was suggested by the discovery that LFA-1 (CD11a/CD18 heterodimer) activation induced adhesion. LFA-1 is an integrin, a cell surface adhesion receptor that facilitates cell-cell adhesion, conjugate formation, and cytolysis (82-84). Upon

activation of a T or NK cell changes in both affinity and avidity drastically strengthen the binding of LFA-1 to ICAM-1, which is necessary for target cytolysis(85). Dustin *et al.* demonstrated that avidity increases upon contact with a target cell by showing LFA-1 clustering at the interface (86). Recent work by Lu *et al.* has shown that conformational change of LFA-1 also results in increased affinity of LFA-1 for ligand (87). LFA-1 also plays a central role in the formation and maintenance of the T cell immune synapse (88), a unique architecture at the interface between a T cell and its target.

Adhesion receptors also play important roles in NK cell activation. LFA-1 blockage with antibody results in blocked cytotoxicity and adhesion to target cells (84, 89). The role of this adhesion receptor is further highlighted by findings that show that increases in local membrane density of ICAM-2 (an LFA-1 ligand), but not total cell surface expression level, results in increased target cytolysis (90). In addition to an intimate role in the formation of the T cell immune synapse, LFA-1 is reported to be centrally located in the recently identified NK inhibitory immune synapse. In contrast to the T cell immune synapse, LFA-1 receptors forms the central core and are segregated from inhibitory KIR enriched areas at the periphery(91). The distribution of LFA-1 at the interface between a target cell and a T cell or NK cell will be discussed in more detail in section 1.5. In summary, LFA-1 is a key adhesion receptor for the generation of NK cytolysis: the increase of LFA-1 adhesiveness, mediated by both affinity and avidity change is requisite for target cytolysis.

1.4 NK Inhibitory Signalling

The first KIR and Ly49 receptors identified were inhibitory receptors, which prevented NK cell cytolysis by recognition of MHC class I on a target cell surface (92). In contrast to the activation receptors that bind ITAM-containing adapter proteins, the NK inhibitory receptors are defined by a long cytoplasmic domain featuring an immunoreceptor tyrosine based inhibitory motif (ITIM) (93). The consensus sequence of the ITIM, I/V/LxYxxL/V, is found in numerous putative inhibitory receptors including inhibitory KIR, Ly49, and NKG2 isoforms. Activation receptors, via the activity of src family kinases, cause the phosphorylation of the ITIM tyrosine residues on the inhibitory receptors (61-63). This provides a docking site for SH2 containing phosphatase-1 (SHP-1), resulting in the activation of the phosphatase and the inhibition of positive signalling (94).

1.41 SHP-1

SHP-1 is the phosphatase that is utilized by ITIM bearing receptors on haematopoietic cells to mediate inhibitory functions. Many lymphocytes possess functional inhibitory receptors that bind SHP-1 including T cells, B cells and monocytes. Although many of these receptors remain uncharacterized, the drastic effects of SHP-1 genetic defects highlight their significance. SHP-1 gene deleted mice (or motheaten mice) die at three weeks of age. Mice with a partial genetic defect of the phosphatase catalytic site, motheaten viable (me^v/me^v), display systemic lymphoproliferative disorder (95).

Although the substrate for SHP-1 has interested many groups, to date there is no consensus for a SHP-1 substrate in human natural killer cells. However, two competing models put forward by Stebbins *et al.* suggest how SHP-1 may function (96). The "permissive dephosphorylation" model suggests that SHP-1 functions by dephosphorylating many membrane proximal substrates. This model is supported by data in which CD16 crosslinking with KIR, colocalizing the two receptors, resulted in reduced phosphorylation of numerous substrates including ZAP70, PLCγ, SLP-76, FcεRIγ and ITAM containing receptors (62, 63). An alternative model is the targeted dephosphorylation of a key cytolytic substrate. Recently, support for this model has grown with reports of targeted dephosphorylation of many key adapter proteins such as pp36 (most likely LAT) and SLP-76 (97, 98). The caveat of both these studies is that dephosphorylation was observed only in the context of an *in vitro* phosphatase assay and the results obtained from these experiments have not been verified *in vivo*.

The best evidence for the relevant SHP-1 substrate in NK cells comes from some currently unpublished findings by C. Stebbins in the Long laboratory. They have communicated to our laboratory that Vav is the substrate of SHP-1 during inhibition of target cytolysis by an NK cell line, YTS (96). To detect the SHP-1 substrates in YTS cells they used KIR/SHP-1 chimeric receptors. SHP-1 was fused to KIR with a deletion in the cytoplasmic tail. The chimeric receptor fused with wildtype SHP-1 had previously been shown to inhibit conjugate formation and tyrosine phosphorylation of an ITAM-containing receptor in conjugates of YTS with MHC expressing targets (63, 84). They constructed two mutations in

SHP-1 to further enhance the sensitivity of their detection methods (99). A trapping mutation, substituting aspartic acid at postion 419 with an alanine, should bind substrate yet prevent catalysis of putative substrate phosphate residues. As a control for non-specific binding a second mutant SHP-1 chimera was utilized. Mutating the arginine to methionine at position 459 completely eliminated substrate binding (100). After retroviral transduction of these constructs into YTS they immunoprecipitated the chimeric receptors from effector:target conjugates. They found that Vav associated specifically with the "trapping" construct, yet was unable to associate with the substrate binding mutant. In addition, they showed that inhibition of SHP-1 activity with pervanadate (a non-specific inhibitor of phosphatases) prevented binding to Vav. Finally, they showed that the binding of Vav to the KIR-SHP-1 chimera is upstream of cytochalasin D inhibition of ITAM-bearing receptor phosphorylation, suggesting that actin polymerization is not required for Vav association with SHP-1. These data suggest that Vav is a direct substrate for SHP-1 in YTS; however, they do not rule out SHP-1 interaction with other substrates in primary NK cells.

1.42 The inhibitory KIR2DL1 receptor

SHP-1 catalysis, activated by binding of the phosphatase to phosphoylated ITIM tyrosine residues, is a key function of inhibitory KIR receptors upon MHC ligation. We undertook our studies in KIR clustering with the inhibitory KIR2DL1 receptor that contains two extracellular Ig domains and an ITIM domain in the cytoplasmic tail. KIR2DL1 is a member of a group of HLA-C specific KIRs that

distinguish between two HLA-C allotypes. Group 1 HLA-C are recognized by CD158a receptors, including KIR2DL1, and are characterized by the amino acids Asn77 and Lys80 at the contact site with KIR. In contrast, group 2 HLA-C ligands contain Ser77 and Asn80 at these positions and bind CD158b receptors.

KIR2DL1 contains a well-characterized zinc-binding motif at the extracellular N-terminus of the receptor. Rajagopalan and Long established that zinc is required for the inhibitory function of this receptor by reversing inhibition with the use of a zinc chelator (101). Further work characterized the H1 motif (HxxH) at the extreme N-terminus as being the region primarily responsible for zinc binding (102). At the time, it was unknown if the zinc binding function was important for binding of HLA, KIR2DL1 multimerization, or association with another cell surface protein. It was thought that this motif was unlikely to be involved in HLA binding as a KIR-Fc fusion protein with a mutated zinc binding motif interacted with HLA-C with affinity equal to that of the wildtype receptor. Fan *et al.* showed *in vitro* that Co^{2+} binding to the KIR zinc binding region resulted in ligand independent dimer formation. In addition they found that the KIR dimers mediated by Co^{2+} occupation of the zinc binding region resulted in higher affinity binding of HLA-C (57). Despite the nonphysiologic nature of cobalt, these data suggested a role for preformed KIR dimers at the cell surface to increase affinity for ligand. Vales-Gomez further explained the function of the zinc-binding region by showing that zinc binding is important for KIR multimer formation (58). This group showed that KIR crosslinking and formation of multimers *in vitro* is controlled by the availability of free zinc and the H1 motif. These data fit well into the published model of the

NK immune synapse where Davis observed inhibition of KIR clustering with the addition of a zinc chelator (91). The work of Davis *et al.* in the context of the immune synapse will be discussed in more detail in section 1.6. Cumulatively, the data discussed here show that zinc binding facilitates multimer formation, enhances ligand affinity and may play an important role in KIR clustering.

The study of inhibitory receptor clustering is in its infancy. In addition to the findings of Davis stated above, some early work has shown that KIR must be clustering with activation receptors to block their function (64, 103). In addition to providing evidence that activation and inhibitory receptor colocalization is necessary for inhibition, these studies highlight a potential link between receptor aggregation and signalling. Furthermore, studies with a mouse NK inhibitory receptor, Ly49A, demonstrated that inhibitory receptors cluster at the interface with target cells (104). These findings suggest that inhibitory clustering maybe an important feature of KIR but the relationship of KIR clustering to signal generation has remained unexplored.

1.5 Receptor Clustering and the Immune Synapse

Outside purely immunological systems, there has been some evidence of the relationship between receptor clustering and signal transduction. The epidermal growth factor receptor (EGFR) is perhaps the best described example in this regard. Early work in this field involved the use of different antibodies to the extracellular portion of the EGF receptor to induce or prevent clustering (105). Schreiber *et al.* pretreated cells expressing EGF receptor with Fab fragments to

various extracellular determinants and attempted to cluster receptors by crosslinking a second antibody to the receptor. They found that some Fab fragments that blocked the clustering of this receptor transmitted only a partial signal, while those with alternate epitopes blocked both clustering and signalling. From these data they concluded that EGFR clustering was necessary for the generation of a competent EGFR signal (105).

1.51 Supramolecular Activation Clusters

Research in the area of T cell receptor clustering came to prominence with the descriptions of the supramolecular activation cluster, or SMAC by Kupfer and colleagues. They observed a unique architecture at the interface between a T cell and an antigen presenting cell (APC) (106). TCR engaged on antigen bearing MHC clusters at the center of the APC interface (c-SMAC), coinciding with the internal localization of key signalling components including PKC-θ, Fyn, and Lck. The peripheral SMAC or p-SMAC is characterized by a ring of adhesion receptors, specifically LFA-1, that surround the central core of antigen receptor. Monks *et al.* also observed that when T cells are presented an altered peptide (antagonist) ligand by APCs, the SMAC fails to form. Based on the coincidence of the formation of the SMAC with activation signalling, they proposed that SMAC formation might play an important role in signal transduction.

Dustin and colleagues expanded on this body of work by observing the interaction of T cells with fluorescent ligands in a planar lipid bilayer. They demonstrated that antigen specific TCR initially forms punctate clusters, while

LFA-1 resides centrally (88). Over time these TCR clusters coalesce into a single coordinated cluster, displacing LFA-1 to the periphery. Grakoui *et al.* explored the effect of MHC density and altered peptide ligands on the dynamic formation of the immune synapse. Mirroring the findings of Monks *et al.*, they found that ligand concentrations capable of initiating T cell signalling also allowed for formation of the mature immunological synapse. Further, they showed that MHC presenting antagonist peptides fail to cause formation of a mature immunological synapse. Although there is a correlation between the ability to form a mature immune synapse or c-SMAC and TCR signalling, they did not observe a signalling event as a direct consequence of synapse formation.

Further correlative evidence suggests that immune synapse formation may initiate signal generation. Publications by Davis show that TCR clustering in live T cell/APC conjugates is coincident with Ca^{2+} release, a second messenger of T cell activation (107). To visualize these features they utilized high-speed imaging to achieve real time analysis of live conjugates. They imaged the dynamic distribution of CD3 and CD4 during T cell conjugation with APC. They observed that CD3 moved rapidly into a cluster at the target interface whereas the CD4 costimulatory molecule moved into the p-SMAC (107).

All of the previous work on T cell receptor clustering had, to this point, only found correlative evidence that linked the ability to cluster receptor to generation of signal. Stronger evidence supporting a link between T cell clustering and activation signalling is presented by data dealing with the T cell phenotype of Mgat5 gene deletion mice (108). Beta 1,6 N-acetylglucosaminyl transferase V

(Mgat5) initiates GlcNAc beta 1,6 branching on N-linked glycans, increasing expression of N-acetyllactosamine, the ligand for galectins. Galectin-3 associates with the TCR and this interaction is dependent upon Mgat5. The Mgat5 gene deleted mice displayed increased TCR dependant signalling and this correlated with increased receptor clustering. The authors also showed that this phenotype can be transferred to wildtype T cells with lactose pre-treatment. Although these authors observed that an increase in TCR signalling correlated with increased clustering, they did not show that TCR clustering is required for signalling to occur.

1.52 Receptor clustering and the cytoskeleton

One of the fundamental questions that arises from these observations is how do receptors move into the interface region in a relatively short period of time? Data in other systems has implicated actin based strategies. Acetylcholine receptor clustering and binding of the receptor to cytoskeletal components is ablated upon disruption of the cytoskeleton with drugs (109). EGF receptor has been shown to be an actin binding protein (110) and is capable of simulating the phosphorylation of myosin motor proteins (111). Wolfing and Davis have studied the role of actin in receptor clustering in T cells by observing the movement of ligand-coated beads toward the T cell:APC interface (112). They used inhibitors of myosin motor proteins to block this process, implicating actin based receptor movement strategies. In summary, when the TCR and other receptors cluster,

they utilize actin-based mechanisms to facilitate receptor movement to the interface with ligand.

1.6 The NK Immune Synapse

To this point all the work was done in receptor systems that generate a positive intracellular signal (ie; growth and/or proliferation). How would inhibitory receptors that activate phosphatases behave at the target cell interface? Using an EGFP tagged MHC ligand, Davis *et al.* characterized the natural killer cell immune synapse that consisted of KIR inhibitory receptors excluded to a ring external to an enriched region in LFA-1 (91). They captured conjugates mounted on a room temperature slide following a twenty minute co-incubation with target cells at 37°C. Davis *et al.* find that MHC clustering is dependent upon ligation with the appropriate KIR receptor. They also demonstrate KIR clustering directly by staining conjugates with fluorescent monoclonal antibody to KIR and LFA-1. Time course data suggested that MHC/ICAM synapses, a putative mirror of KIR/LFA-1 were remarkably stable, lacking large structural changes for periods up to 22 minutes post incubation. Against a panel of inhibitors including azide, an ATP inhibitor, and cytochalasin D, an inhibitor of f-actin, this group found no effect on KIR clustering. The only inhibitor that was observed to affect KIR mediated MHC clustering was phenanthroline, a zinc chelator. As noted previously, zinc binding has been observed to functionally affect KIR signalling and enhance KIR multimerization in an *in vitro* system (58, 102).

1.7 Hypothesis to be tested

In this study we wanted to uncover more about the mechanism and consequences of inhibitory KIR clustering. Studies with EGFR receptor have linked clustering with signalling (105). In addition, competent receptor signalling is correlated to receptor clustering of the TCR (107). The contribution of inhibitory receptor clustering has not been identified and we hypothesized that:

KIR clustering is required for KIR signalling.

The initial study by Davies *et al.* detailing the architecture of NK immune synapse found that KIR-mediated MHC clustering was independent of actin cytoskeleton and cellular ATP. Both the actin cytoskeleton and a source of cellular ATP are required for activation signalling (113). From these results we deduced our second hypothesis:

KIR clustering is independent of signal transduction.

Finally, the dissolution of the T cell immune synapse is thought to be controlled by a number of factors including chemokine gradients, antigen affinity, and inhibitory receptor signalling (114, 115). Clustering of inhibitory receptors (ie; KIR), may affect the formation and dissolution of the activation immune synapse. A logical point of signal integration is the actin cytoskeleton. Our final hypothesis is:

KIR clustering affects the actin polymerization.

Chapter 2. Materials and Methods.

2.0 Antibodies and Cell Lines.

Monoclonal antibodies EB6 (anti-CD158a) and MOPC21 (IgG1) were used to monitored receptor expression and obtained from Coulter-Immunotech and Sigma. FACS was regularly preformed on cell lines to ensure a stable level of receptor expression. KIR2DL1 staining was performed with primary staining of $3x10^5$ cells with 1μg EB6 antibody (Immunotech-Coulter). Phychoerythrin (PE) goat anti-mouse secondary antibody (Cedarlane, Toronto ON) was added and analyzed in a FACScan (Becton-Dickson). The HB202 hybridoma producing antiLFA-1 (IgG1) was obtained from ATCC. D. Burshtyn purified anti-LFA-1 antibody on a protein A column. Direct labelling of EB6 with ALEXA568 fluorochrome was performed with the ALEXA568 protein labelling kit, according the manufactures instructions (Molecular Probes, USA).

The NK92 cell line was provided by Hans G. Klingemann and maintained in 50% Myelocult (Stem Cell Technologies, Vancouver BC) and 50% IMDM with 7.5% Characterized FCS (Hyclone) and 100 U/ml rIL-2 (Tecin, NCI, USA). The YTS-EcoR1 subline was obtained from G. Cohen and maintained in IMDM 15%FCS with 2mg/mL G418. 721.221, 221-Cw3, and 221-Cw4 have been described (116).

YTS stable transfects were generated by Mary Peterson at NIH (Rockville, MD) using the previously described method (26). Briefly, Bosc23 retroviral packaging cells were transfected with 10μg of the plasmid pBABE containing the EGFP-KIR chimeras. Supernatant was harvested 36 hours later and incubated

with YTS-EcoR1. Starting at 48hrs post infection puromycin was titrated over 4 days into a final concentration of 1μg/mL. After 10 days, cells were analysed for EGFP expression by flow cytometry. At the University of Alberta subcloning and FACS further sorted populations for various levels of receptor expression. Subclones were screened for the ability to lyse 721.221 cells.

2.1 Construction of KIR-EGFP Chimeras.

These constructs were generated at NIH (Rockville, MD) by Deborah Burshtyn and Mina Sandusky. The vector EGFP-N2 was purchased from Clontech (Palo Alto, CA). To prevent internal translation initiation of the EGFP from the chimeras the start codon ATG (methionine) of EGFP was mutated to ATA (isoleucine) by the QuikChange™ Site Directed Mutagenesis Kit (Stratagene, LaJolla, CA) using the primer CCAACAGATATCATCGTCTACACGGAACTTCC and the complimentary primer. The coding regions were sequenced to ensure only the desired mutation was incorporated. The resulting construct was named pEGFP-N2ML and pEGFP-N3ML.

KIR2DL1-TG was created by fusing EGFP to the C-terminus of the cytoplasmic tail of 2DL1. First, the BsrG1 site for 2DL1 was removed with a silent mutation. Second, the stop codon of 2DL1A in pSport was mutated to contain a Xma1 site using the forward primer GTTGTCTCCTGCCCGGGAGCACCACAG TCAGG and its compliment to generate 2DL1AX. The EcoR1-Xma1 fragment of 2DL1AX was isolated and cloned into the Xma1-EcoR1 fragment of pEGFP-N3 with the methionine to isoleucine mutation to create 2DL1-TG. 2DL1-TG encodes

the entire coding sequence of KIR2DL1 linked to EGFP by the amino acids GSIAT.

To construct KIR2DL1-HG two inframe fusion proteins with EGFP were generated first. First, 2DL1 was cloned into the Sal1 and Not1 site of pEGFP-N1. Next, the signal sequence of 2DL1 was fused upstream of the EGFP by cloning the SnaB1-Msc1 from 2DL1-EGFP-N1 into SnaB1 and Msc1 of pEGFP-N2-ML. The linker between the signal sequence and EGFP is IHRPVAT. Next, the extracellular, transmembrane and cytoplasmic tail of 2DL1 was fused downstream of EGFP by cloning MscI to HindIII fragment from 2DL1A-pSPORT into the Ecl136 and HindIII sites of pEGFP-C2 which generates the linker sequence VSPLE. Finally, to generate the final chimera, the BsrG1 to Not1 fragment from 2DL1A in pEGFP-N2 was cloned into the BsrG1 and Not1 sites of the pEGFP-N1 backbone with the signal sequence. All constructs were confirmed by sequencing. The 2DL1-HG and 2DL1-TG chimeras were subcloned into pBABE using EcoR1 and Not1 sites of pBABE (provided by G. Cohen).

2.2 Recombinant Vaccinia

Recombinants prepared by D. Burshtyn and M. Weston (NIH, Rockville, MD). CV-1 fibroblasts were infected with vaccinia virus strain WR and transfected with pSC66 plasmid containing the KIR-EGFP chimeric receptors. CV-1 cells were infected for 2 h. at 85% confluence with $2.5x10^6$ pfu of virus. Using lipofectin (Invitrogen, Burlington, ON) infected CV-1 cells were transfected overnight with 15 μg pSC66 plasmid carrying KIR chimeras. 50 h. post infection

cells were harvested and subjected to three freeze/thaw cycles, purified and plated on TK- plates. Recombinants were selected for growth in bromodioxyuridine, expression of beta-galactosidase (βgal) and the protein of interest. Individual blue (βgal+) plaques were isolated and reselected.

Recombinant virus was expanded in confluent BGMK cells (provided by M. Barry). Confluent monolayers of BGMK cells were infected with $1x10^8$ pfu of crude virus stock in 10 mL serum free DMEM (infection media). After a 2 h. infection, rocking each flask every thirty minutes, 30 mL of DMEM containing 12% Newborn Calf Serum (Invitrogen, Burlington, ON) was added. After 48-72 h., when the cytopathic effects of the virus were visible by microscope (ie; non adhesive cells, rounding up), cells were harvested and resuspended in OPTI-MEM (Invitrogen, Burlington, ON). Cells were subjected to three freeze/thaw cycles, homogenized, the nuclei pelleted, and the remainder sonicated for 1 minute. Vaccinia virus was purified by ultrcentrifugation at 13,500 rpm for 80 minutes at 4°C. Pelleted virus was resuspended in 10mM Tris at pH9.0 and stored at –70°C.

2.3 Biochemical Inhibitors

PP2, piceatannol, rottlerin, LY29402, and cytochalasin D were all dissolved in DMSO and stored according the supplier's instructions (Calbiochem). 1,10 phenanthroline (Sigma), a chelator of zinc, was dissolved at 2 M in ethanol and prepared fresh on the day it was to be used. Sodium azide

(Sigma) was also prepared fresh on the day it was to be used and was dissolved in ddH20 to a final concentration of 3 M.

2.4 Cytolysis Assays.

For BATDA release assays, the target cells were pre-labelled with BATDA reagent (Wallac), washed, and incubated with the appropriate ratio of effector cells for 2 hrs at 37°C with 5% CO_2. All cells displayed over 95% exclusion of trypan blue at the time of assay and were in log phase of culture. The supernatant concentration of BATDA reagent was measured by chelation with europium development solution (0.4 M acetic acid, 0.1 M europium standard, pH 4.0) that allowed for time resolved fluorescence of europium to be read on a plate reader (Wallac) (117). Percent cytolysis was calculated with the formula below:

Percent cytolysis = ((release of sample – spontaneous release)/ (maximum release – spontaneous release)) x 100.

Cytolysis assays were also performed using ^{51}Cr release. Vaccinia virus infection and cytolysis assays, were essentially as described (118). In brief, infection of vaccinia virus took place at various pfu, as indicated in figure legends in 500 µl of warm Infection media (IMDM, glutamine, nonessential amino acids (Invitrogen, Burlington, ON)), 0.1% bovine serum albumin, 100U/mL IL-2) resulting from a 1:1 mixture of 2x diluted virus stock to 2x cells. After an immediate vortex, the cells coincubated with virus for 1.5 hrs at 37°C with 5%

CO_2. During this time period $1x10^6$ target cells were labelled with 1mCi of ^{51}Cr (NEN) for 1hr and subsequently washed in warm CTL media (IMDM, 5% FCS, glutamine). In triplicate, $5x10^3$ target cells were combined with the appropriate number of effectors, based on E:T, and incubated at 37°C with 5% CO_2 for 4hrs. Results were read by γ-counter and percent lysis calculated as described above.

2.5 Conjugate Adhesion Assay.

The conjugate adhesion assay has been previously described (84). Target and effector cells are labelled with 0.16 M of PKH26 (red) and PKH67 (green) membrane dyes (Sigma) respectively. The dye reaction was halted with the addition of 1 mL of FCS for 1 minute and samples subsequently washed. Cells were rested at 37°C for a 1 hour prior to incubation to allow stabilization of dye. Immediately prior to combining of effector and target cells, samples were cooled on ice to 4°C. $1x10^5$ target and $2x10^5$ effector cells were spun at 25xg at 4°C and placed at 37°C to allow for conjugation to occur. After incubation the sample was quickly vortexed and immediately fixed with 1% paraformaldehyde. The data were analyzed by FACScan (Becton-Dickson). Percent YTS in conjugates was calculated with the formula below:

Percent conjugates = (two color events)/ (total effector events) X 100

2.6 Confocal Imaging of Live Conjugates.

Confocal imaging of conjugates has been done as previously described (119). Conjugates were imaged live in a modified 60mm petri dish. The dish was drilled with 0.75 cm diameter hole in the bottom and a size 1 coverslip was glued overtop with epoxy. $1x10^5$ effector and $2x10^5$ target cells were mixed, subjected to a low speed spin and placed in the 60mm petri dish with 2mL of complete media overlayed with 1ml embryo tested mineral oil (Sigma) for immediate analysis. Analysis of conjugates took place on a temperature controlled stage at 37°C with 5% CO2. The conjugates were captured with a Zeiss 310 Axiovert Confocal Microscope, and the data was subsequently analyzed by Zeiss 510 Image and Metamorph 4.0 (Universal Imaging) Software packages. Images were collected with a pinhole of 1U, 12-bit resolution, and a z-stack slice interval of 0.5μM. Heteroconjugates were identified by the presence of an EGFP fluorescing YTS effector cell and an unlabeled, or red labelled (PKH67, Sigma) target cell. The criterion for conjugate inclusion was that it consisted of a heteroconjugate that was obviously adhered. All thresholds were set to minimize saturation of the detectors while still allowing detection of EGFP positive cells. Isolated interface regions were created by extracting raw data from the LSM image file into the Metamorph program. There a mid section slice was selected and the interface region carefully traced. The image in this region was duplicated and reconstructed into a 3D projection. The projection was subsequently rotated to allow direct visualization of the interface. Pseudocolor is used to indicate intensity of the cluster, white/red - high intensity thru blue/purple – low intensity.

2.7 Confocal Imaging of Fixed Conjugates

Conjugates were formed similar to the method developed for the conjugate assay. $1x10^5$ targets were combined with $2x10^5$ effectors and incubated at 37°C for the appropriate time course. Unless otherwise noted conjugates were incubated for 20 minutes. After incubation, samples were immediately fixed with cytofix/cytoperm (BD Pharmingen) for 20min at 4°C. Samples were then wash with Perm/Wash Buffer (BD Pharmingen) and then incubated with the appropriate directly labelled antibody (ie; ALEXA 568 EB6), at saturating concentrations determined by FACS staining, for 30 min at room temperature. Samples were again washed and then analyzed by confocal in a modified 60mm dish (as above) overlaid with 3ml D-PBS.

Chapter 3. KIR signalling is not dependent on KIR clustering

3.1 Introduction

The regulation of NK cytotoxicity has historically been described by the missing self hypothesis (120). Karre proposed that NK cells recognize their targets by a lack of self MHC class I expression. In humans, these inhibitory receptors are members of either the C-type lectin or the Ig-like superfamily. The CD94 C-type lectin receptors are highly conserved between species(36); however, the polymorphic KIR receptors are responsible for greater cytolytic inhibition. As an inhibitory member of the KIR family, KIR2DL1 contains a cytoplasmic ITIM domain which binds and activates SHP-1, blocking downstream effector functions such as MTOC reorganization and granule release(121). On its extracellular domain, KIR2DL1 contains two Ig domains which together allow MHC binding and a zinc-binding motif at the N-terminus which is important for receptor dimerization and signal generation(58, 101, 102).

As discussed in section 1.5, in T cells, the cartography of adhesion and activation receptors at the interface between target and effector cells has been described as peripheral and central supramolecular activation clusters(106). Monks *et al.* described a structure in which the TCR antigen receptors reside in a central core surrounded by a ring of the adhesion molecules, LFA-1 and talin. The dynamic interplay between these groups of receptors has been termed by Dustin and colleages the "immunological synapse"(88). They have found that adhesion receptors preliminarily exist in the central core of the interface and subsequently become disrupted by TCR/MHC pairs moving toward the center of

the contact region. Despite data showing that antagonistic peptide fails to cluster receptor, no work has conclusively shown if these supramolecular activation clusters are required to initiate a signal. The NK cell "immunological synapse" has been roughly described by Davis *et al.*(91). His group found LFA-1 adhesion receptors clustered at the center of an inhibitory KIR ring.

Recently, activating KIR receptors have been characterized that recognize the MHC class I(122). Many of these activation receptors have similar ligand binding regions to their inhibitory counterparts. With similar receptors performing either the inhibitory or activating functions, how is inhibition of cytolysis achieved? A hypothesis that arose from this conflicting dichotomy suggested that the higher affinity inhibitory receptors were able to initiate clustering when engaged on ligand facilitating signal generation. To test this hypothesis we generated EGFP fusion proteins with the inhibitory KIR2DL1 receptor (Figure 3). Three constructs were generated and named: KIR2DL1-TG with the EGFP moiety at the C-terminus of the cytoplasmic tail, KIR2DL1-HG with EGFP proximal to a zinc-binding motif at the extracellular N-terminus, and KIR2DL1-MG in which EGFP was fused to a cytoplasmic tail truncation which deleted the ITIM.

3.2 Characterization of chimeric receptor function in YTS.

We wanted to explore the receptor dynamics of KIR at the interface with live target cells. To date, a method for stable transfection of NK cell clones remains to be successfully achieved; however, retroviral mediated gene transfer into an NK-like cell line, YTS, has been described (26). The YTS line does not

express any known KIR and is naturally cytolytic against MHC class I negative targets. Utilizing retroviral transduction we generated YTS lines expressing the KIR-EGFP chimeric receptors. These lines were subcloned, and subsequently sorted for equivalent levels of receptor expression (Figure 4).

The EGFP domain was proximal to either the ITIM region (KIR2DL1-TG) or to the zinc-binding motif (KIR2DL1-HG), and we needed to ensure that placement of the EGFP domain in these regions did not interfere with receptor function. We tested the ability of the transductants to inhibit YTS cytotoxicity, employing a BATDA release assay to measure the ability of these cell lines to lyse various target cell lines. BATDA reagent is retained within the prelabelled target cell and upon lysis is released into the supernatant. When combined with europium, BATDA release can be quantified through time resolved fluorometry. All cell lines were capable of lysing targets expressing non-protective HLA-Cw3. Both chimeric receptors containing the full length KIR were able to effectively inhibit lysis as well as the transductant expressing wildtype KIR2DL1 when combined with target cells expressing protective ligand, HLA-Cw4 (Figure 5a, 5b, and 5d). As expected, the construct from which the ITIM region was deleted, 2DL1-MG, was unable to protect HLA-Cw4 bearing target cells from cytolysis (Figure 5c). These data show that the EGFP construct does not interfere with the inhibitory function of KIR in the YTS cell line, and that the signal transmitted is competent to block cytolysis of target cells.

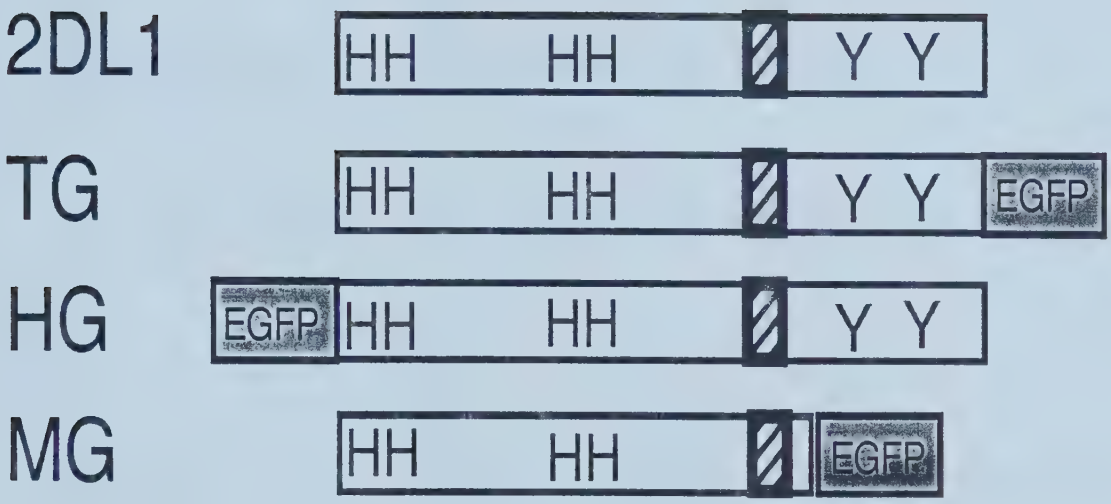


Figure 3. *Schematic of KIR-EGFP fusion proteins.* For comparison the wild type KIR 2DL1 is shown. Open boxes represent KIR2DL1 sequence, shaded boxes represent the EGFP moiety, and the crosshatched region the transmembrane region.

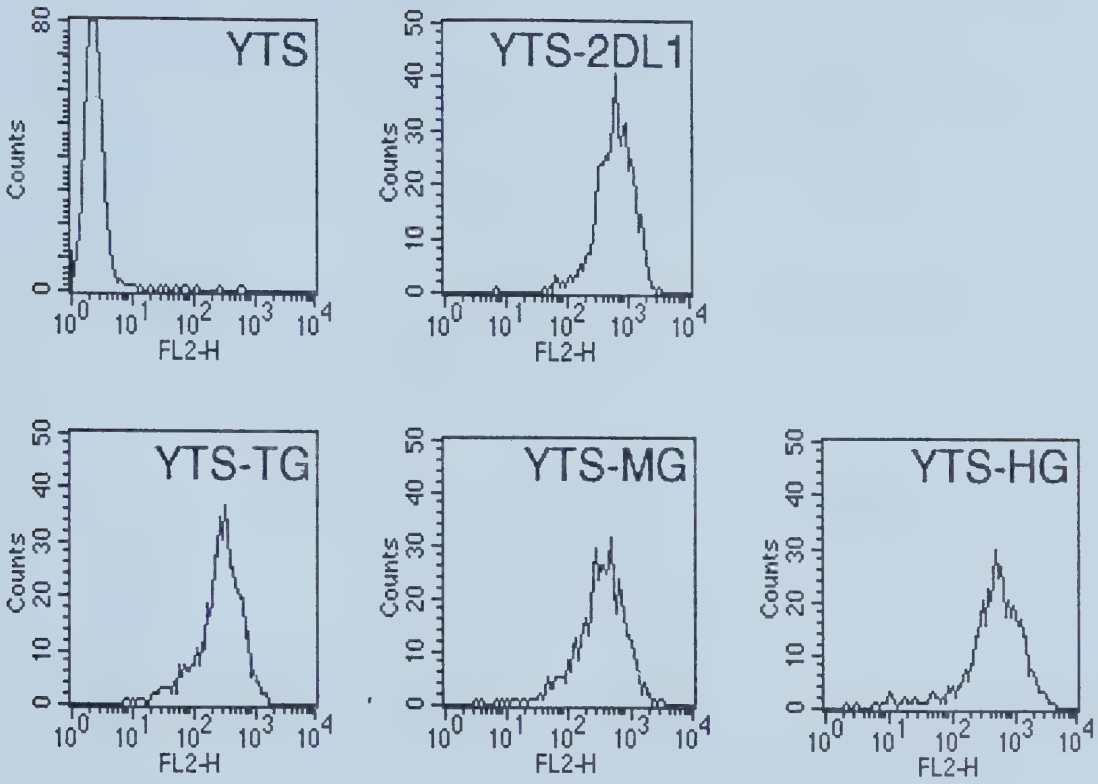


Figure 4. *Receptor Level of Isolated YTS lines expressing KIR-EGFP.* YTS cells retrovirally transduced with cDNA encoding the KIR-EGFP chimera were subcloned and sorted by FACS staining with the EB6 antibody specific for KIR 2DL1. Sublines were selected for equivalent levels of receptor expression.Receptor expression of representative sublines are depicted above.

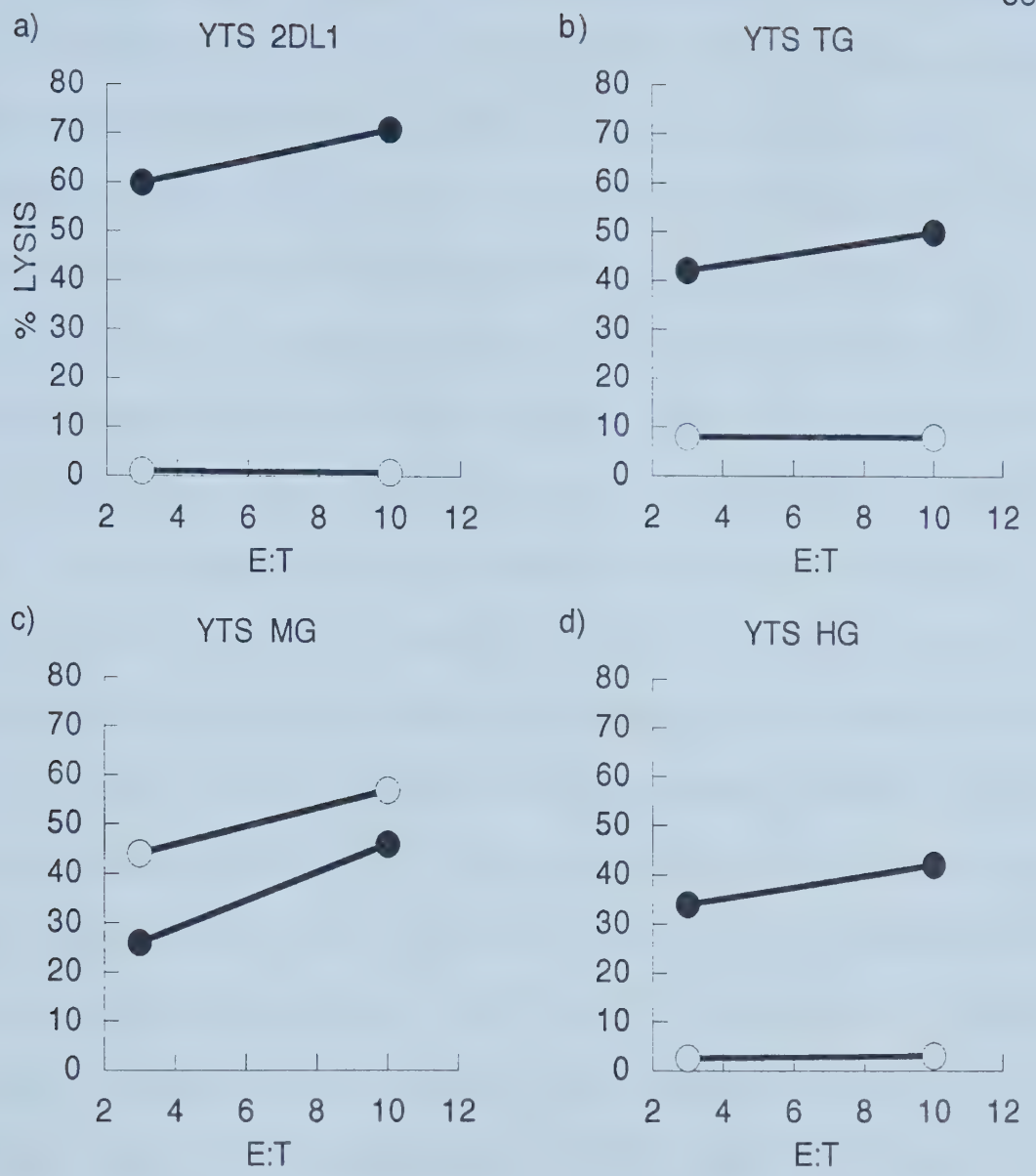


Figure 5. *Receptor mediated inhibition of YTS cytotoxicity*. The YTS lines were tested for the ability to lyse protected (221-Cw4, open circles), and unprotected (221-Cw3, filled circles) targets in a BATDA release assay.

Another function of inhibitory KIR in YTS lines is the reduction of strong LFA-1 mediated adhesion to MHC protected target cells(84). During lymphocyte activation tyrosine kinase activity leads to inside-out signalling, namely the increase of affinity and avidity of LFA-1 for ICAM-1 (85). Our laboratory had previously shown that KIR, by activating SHP-1, could block this process in YTS lines transduced with inhibitory KIR receptors (84). We utilized a two color conjugate assay to determine if this process was conserved for the KIR-EGFP chimeric receptors. Target cells were labelled red with a nonspecific membrane dye, PKH67, and effector cells were labelled green with PKH26. Targets and effectors were combined, incubated for a time course, vortexed and fixed with 1% paraformaldehyde. Data was collected by measuring two color events over total effector events by FACScan. As with the wildtype receptor, we found that the full-length receptor constructs, 2DL1-TG and 2DL1-HG, reduced adhesion to target cells, whereas the ITIM deletion mutant, 2DL1-MG, did not decrease adhesion to targets cells which express correct ligand (Figure 6). These data again show that the EGFP receptors transmit a competent inhibitory signal, confirming our findings in figure 5, and extending them to inhibitory signals that disrupt adhesion.

To ensure that these responses were typical of other naturally cytotoxic cells we also observed receptor function in NK92 using a vaccinia virus mediated gene transfer system(123). Recombinant vaccinia were prepared which contained the chimeric receptors under control of an early gene promoter. NK92 cells were infected for 1.5 h and then mixed with target cells in a 2 h BATDA

release assay. In NK92, the full-length receptors inhibited lysis whereas the 2DL1-H1 receptor (A previously characterized receptor with a mutation in the zinc binding region) was deficient (Figure 7). This confirms our YTS data in an alternative cell line and shows that EGFP has not significantly altered the expected function of the receptor at high levels of receptor expression. Regarding the processes of adhesion, cytolysis and receptor clustering, we are confident that YTS functions similar to the NK92 cell line (see Appendix I) and it is representative of the activities in primary cytotoxic cells.

3.3 2DL1-HG required more receptor to transmit an inhibitory signal.

Early data in EGFP chimeric lines, at unmatched levels of receptor expression, suggested that low levels of 2DL1-HG receptor were unable to inhibit adhesion. To ascertain if 2DL1-HG exhibited a bona fide defect in signaling we exploited a feature of the vaccinia expression system. Using superinfection, the capability of vaccinia viruses to infect a single cell multiple times, we titrated the level of receptor expression to explore the function of the receptors at low levels in the NK92 system. We titrated both the 2DL1-TG and 2DL1-HG receptor in NK92 and observed the ability of these receptors to mediate inhibition of cytolysis in a BATDA release assay. The 2DL1-HG receptor was unable to mediate the same degree of target cell protection, measured by relative lysis, as the 2DL1-TG receptor at low receptor levels (Figure 8). This suggests that despite competent inhibitory signaling the 2DL1-HG receptor is unable to generate an inhibitory signal to the same extent as the 2DL1-TG receptor.

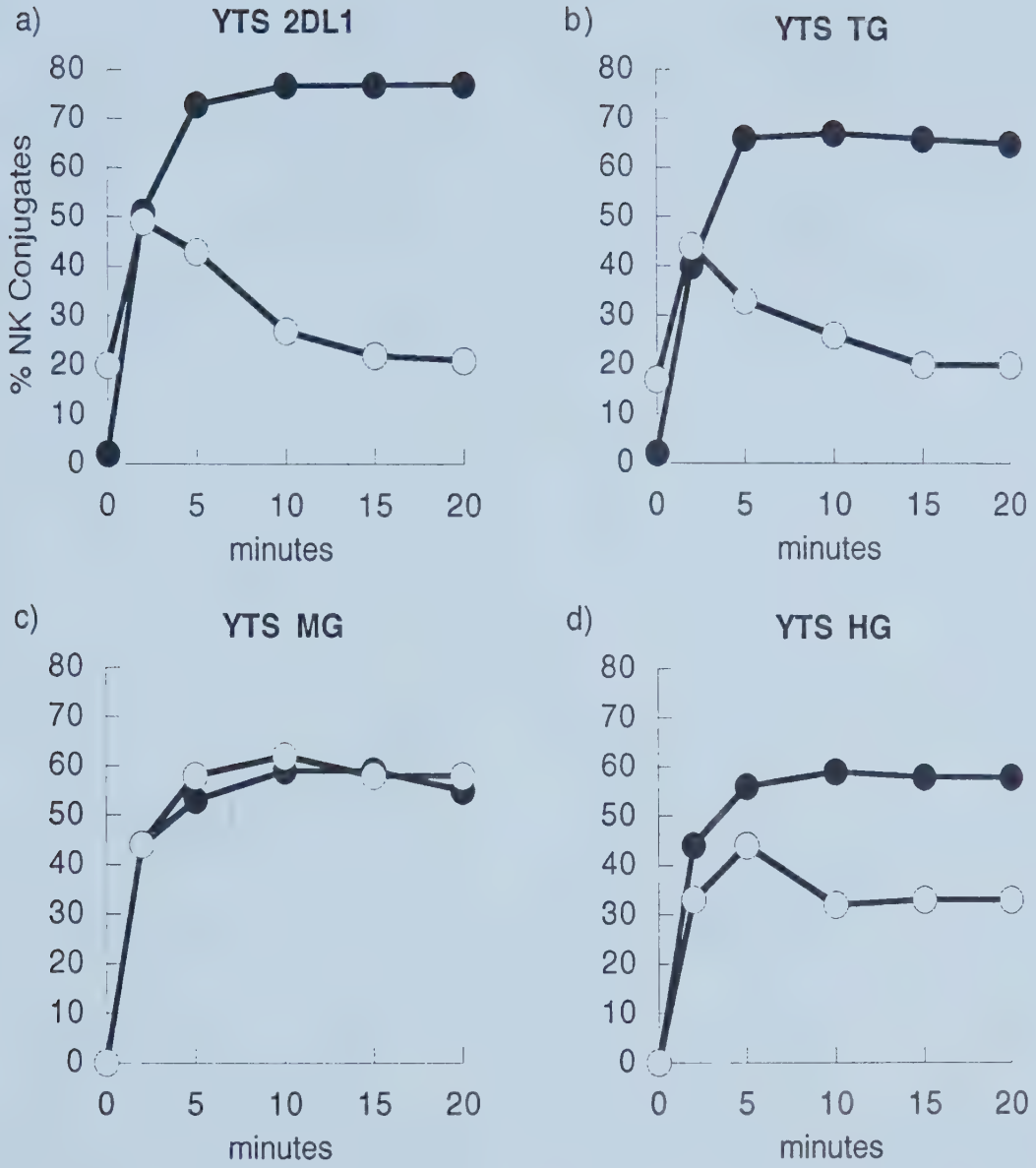


Figure 6. *Reduction of Conjugate Formation by KIR-EGFP chimeras.* Matched YTS sublines were assayed by two-color conjugate formation assay. YTS lines were labeled with PKH67 (Green) and target cells (221-Cw3, filled circles and 221-Cw4, open circles) were labeled with PKH26 (Red) non specific membrane dyes. Targets and Effectors were combined at 4°C and incubated at 37°C for the indicated times.

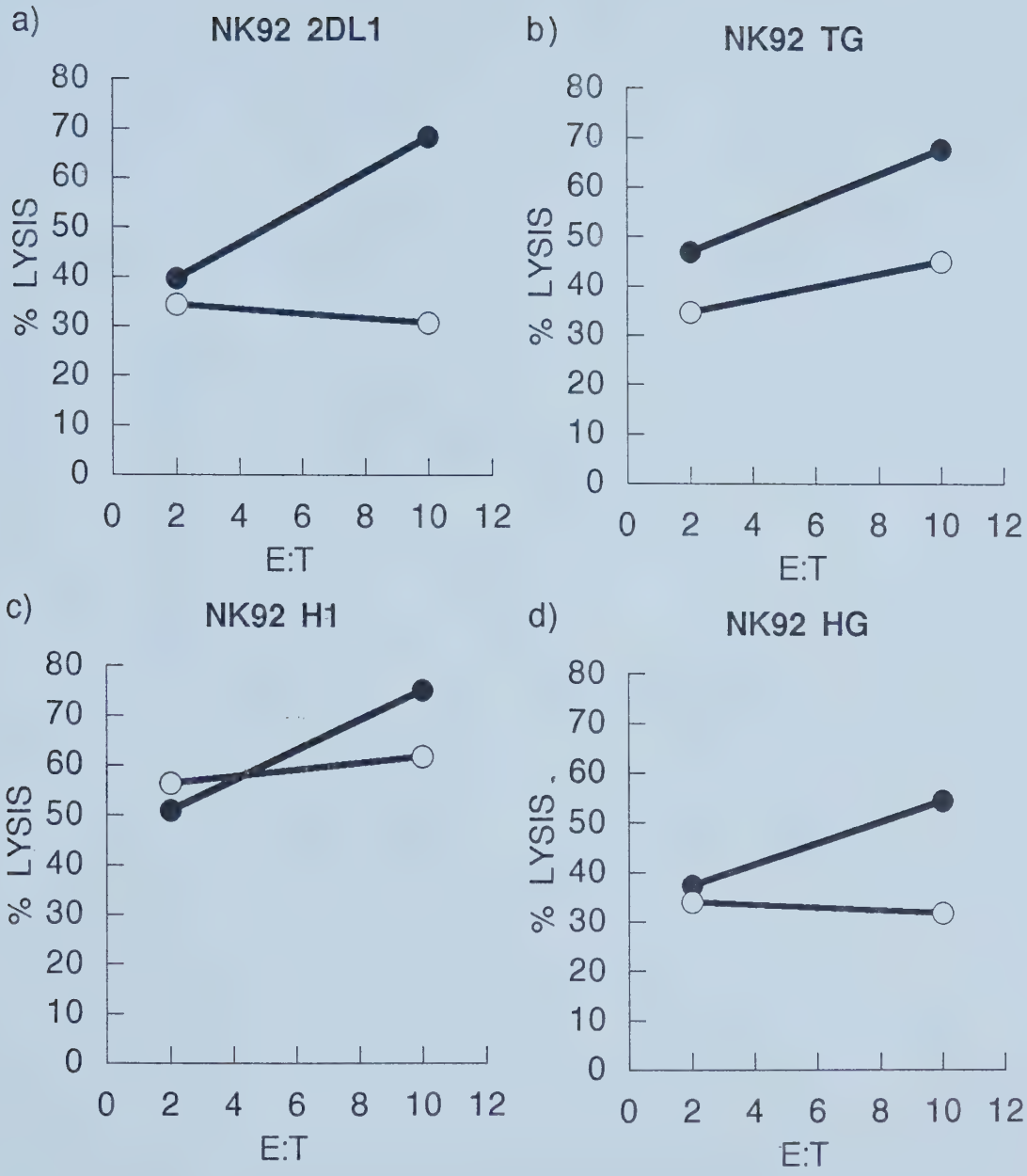


Figure 7. *Receptor mediated inhibition of NK92 cytolysis*. NK92 cells were infected with vaccinia virus carrying the indicated receptors at the following pfu/cell and MFI respectively: 2DL1,10, 127; H1,15, 108; HG, 40, 84; TG, 25, 81. The receptor levels were determined by flow cytometry using EB6 and PE-conjugated secondary antibody. Killing of 221-Cw3 (filled circles) or 221-Cw4 (open circles) target cells was measured by BATDA release assay.

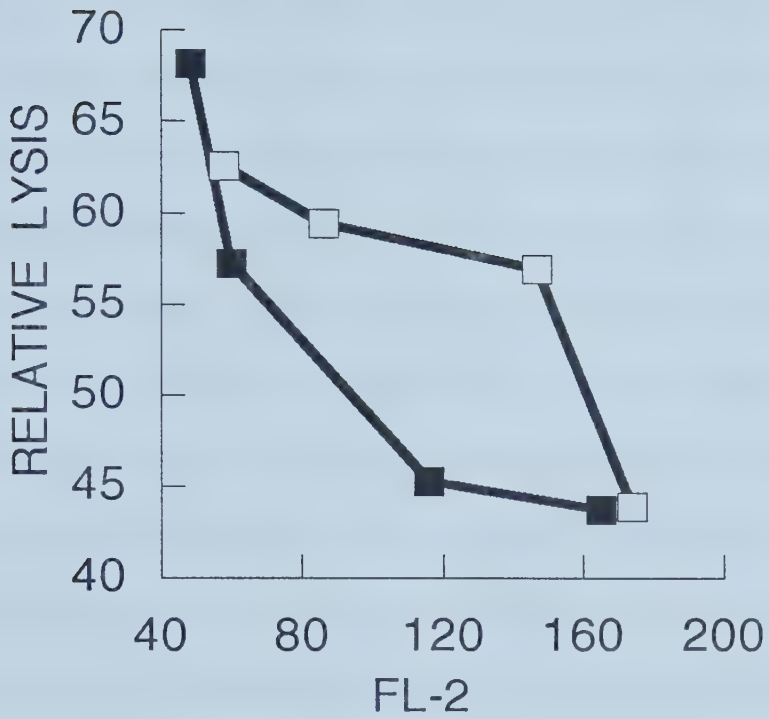


Figure 8. *Comparison of the ability of 2DL1-TG and 2DL1-HG to inhibit cytolysis.* NK92 were infected with 5, 10, 25, and 50 pfu/cell of vaccinia carrying TG (filled boxes) or 4, 8, 16, and 32 pfu/cell of vaccinia virus carrying HG (open boxes). The relative lysis of 221-Cw4 compared to 221-Cw3 is plotted against receptor expression measured by EB6 staining. The lysis was measured using the BATDA release assay.

3.4 2DL1-HG and 2DL1-TG both require zinc to function.

One hypothesis to explain the 2DL1-HG signaling deficiency may be an altered ability to use the zinc-binding motif, which is proximal to the EGFP fusion site in the 2DL1-HG receptor. The zinc-binding motif is implicated in receptor dimerization and is required for inhibitory signal generation(58, 101, 102). If zinc-binding was altered and 2DL1-HG signal transduction was modified by the addition of the N-terminal EGFP, we expect that the addition of a zinc chelator would not effect the function of this receptor. We tested this hypothesis by addition of the zinc chelator 1,10 phenanthroline to a 4 h chromium release assay. We labeled target cells with chromium-51, and then combined these with NK92 cells that had been infected with vaccinia encoding 2DL1-TG or 2DL1-HG receptors. Chromium release from lysed target cells was read in a γ-counter. We observed that 1,10 phenanthroline reverted receptor mediated inhibition for both the 2DL1-TG and 2DL1-HG receptor (Figure 9). Contrary to our initial hypothesis, 2DL1-HG continued to require zinc to potentiate an inhibitory signal.

3.5 Receptor clustering of 2DL1-TG and 2DL1-HG.

The 2DL1-HG receptor shows a deficiency in signaling at low receptor levels when compared to the 2DL1-TG receptor despite 2DL1-HG's ability to transduce an inhibitory signal. We hypothesized that this defect may be manifested in the ability of the receptor to cluster in response to ligand. Experiments by other groups had shown that KIR was able to cluster EGFP tagged MHC ligand and that this process was sensitive to treatment with 1,10

phenanthroline (91). We examined live conjugates by confocal imaging of a z-series from each conjugate and reconstructed the interface region using Metamorph imaging software. Conjugates were formed by a low speed spin and placed in a 35mm dish with media at 37°C and 5%CO_2 to allow visualization at physiologic conditions. Data was collected at a pinhole of 1U and a z-stack distance of 0.5μm (see section 2.5 for more details). When combined with unprotected targets, YTS-TG failed to cluster (Figure 10, top). Conjugates of YTS-TG with protected targets showed a large increase in the amount of KIR2DL1 at the target:effector interface (Figure 10, bottom). When the interface region was reconstructed we observed that KIR formed a large circular aggregate at the interface between a YTS and the target cell. This finding confirms a previous report of KIR mediated EGFP-MHC clustering(91). However our report differs in that we fail to observe an absence of KIR-EGFP fluorescence in the middle region of the KIR cluster, an area where Davis *et al.* have observed LFA-1 enrichment and implied KIR exclusion.

When we placed YTS-HG in this system it failed to visibly cluster receptor in large macromolecular aggregates while conjugate with both protected and unprotected target cells (Figure 11). This confirmed our hypothesis that the deficiency in the ability of 2DL1-HG receptor to signal was coordinate with defective receptor clustering. Notably, these data together with the results discussed previously show that KIR clustering is not required for the generation of an inhibitory signal. In addition, we add to previous findings showing although KIR clustering may be zinc dependant, this process is upstream and separate

from the multimerization of the receptor into large macromolecular aggregates at the target:effector interface.

As only a single previous report demonstrated that KIR clusters when engaged on ligand(91), we needed to ensure that the clustering observed with the 2DL1-TG receptor was phenotypically similar to that of the wildtype receptor. We stained fixed conjugates with mAb EB6 which had been directly conjugated with the ALEXA 568 fluorochrome. This allowed independent visualization of both the EGFP and ALEXA 568 fluorescence by confocal microscopy with excitation/emission wavelengths of 488/515 and 568/610 respectively. YTS expressing the wildtype KIR2DL1 clustered at the interface in a manner similar to that observed by EGFP fluorescence in the YTS-TG line (Figure 12). To further confirm these findings, YTS-2DL1 (wt), YTS-TG and YTS-HG conjugates with 221-Cw4 were stained with EB6-ALEXA 568. Antibody staining and EGFP fluorescence colocalized at the cell membrane and at the contact region of the conjugates (Figure 13), confirming the specificity of our antibody and our findings with the 2DL1-HG receptor.

3.6 Summary of Results

The results detailed in this chapter consist of three major findings. First, addition of an EGFP moiety at either C or N terminus does not interfere with KIR inhibitory signaling. Shown in figures 5 through 7, in two different cell lines, the KIR-EGFP receptors are competent in both the NK92 and YTS cell lines to signal to inhibit adhesion and cytolysis. In addition to inhibitory signal generation, both

receptors retain the requirement for zinc binding, despite proximity of the 2DL1-HG receptor to a zinc binding motif (Figure 9). Second, 2DL1-HG receptor is unable to mediate a strong inhibitory signal at low receptor levels. As shown in figure 8, when receptor level was titrated in the NK92 vaccinia system, we found that the YTS-HG receptor fails to provide the same degree of protection from cytolysis at low receptor levels. Thirdly, 2DL1-HG, despite competent inhibitory signaling, fails to cluster receptor in a macromolecular complex. Outlined in figures 10 through 13, this together with the prior work in this chapter establishes that KIR clustering is not required to mediate an inhibitory signal.

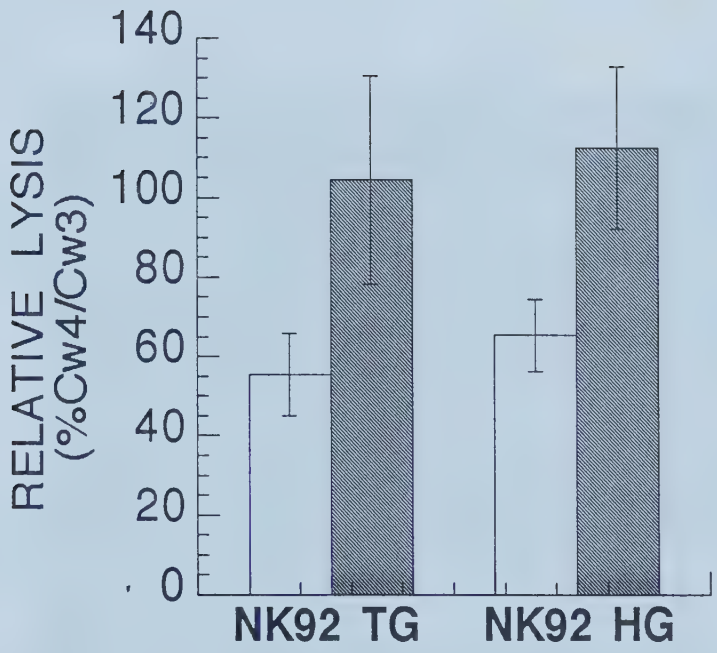


Figure 9. *Effect of 1,10-phenanthroline on KIR mediated inhibition.* KIR2DL1-TG and KIR2DL1-HG receptors were tested for the ability to inhibit lysis in the presence (shaded bars) and absence (white bars) of 1mM 1,10-phenanthroline. The NK92 cells were infected at 20pfu/cell and assayed for lysis of 221-Cw3 or 221-Cw4 cell lines in a chromium release assay. The relative lysis for Cw4 to Cw3 is depicted for an E:T ratio of 10:1.

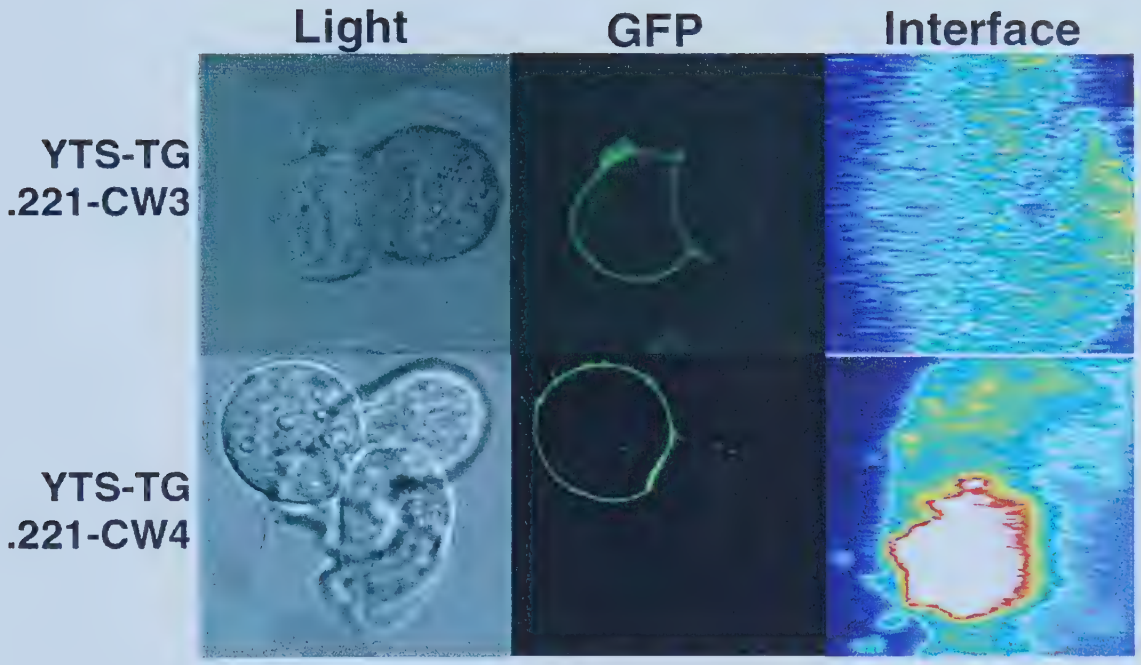


Figure 10. *Ligand-induced clustering of the KIR2DL1-TG chimera*. YTS-TG cells were combined with unprotected (221-Cw3, top) or protected (221-Cw4, bottom) target cells. Images of conjugates were collected as transmitted *light* and confocal *EGFP* fluorescence. The right panel depicts a reconstructed Z-stack taken through the vertical plane of the interface between the target and effector cell at the point of contact. The fluorescence image at the *interface* region was reconstructed using Metamorph imaging software. The fluorescent intensity scale is purple (low) → white (high). Between 20 and 50 images were collected for each group during multiple experiments at various time points during the initial 30 minutes post-conjugation.

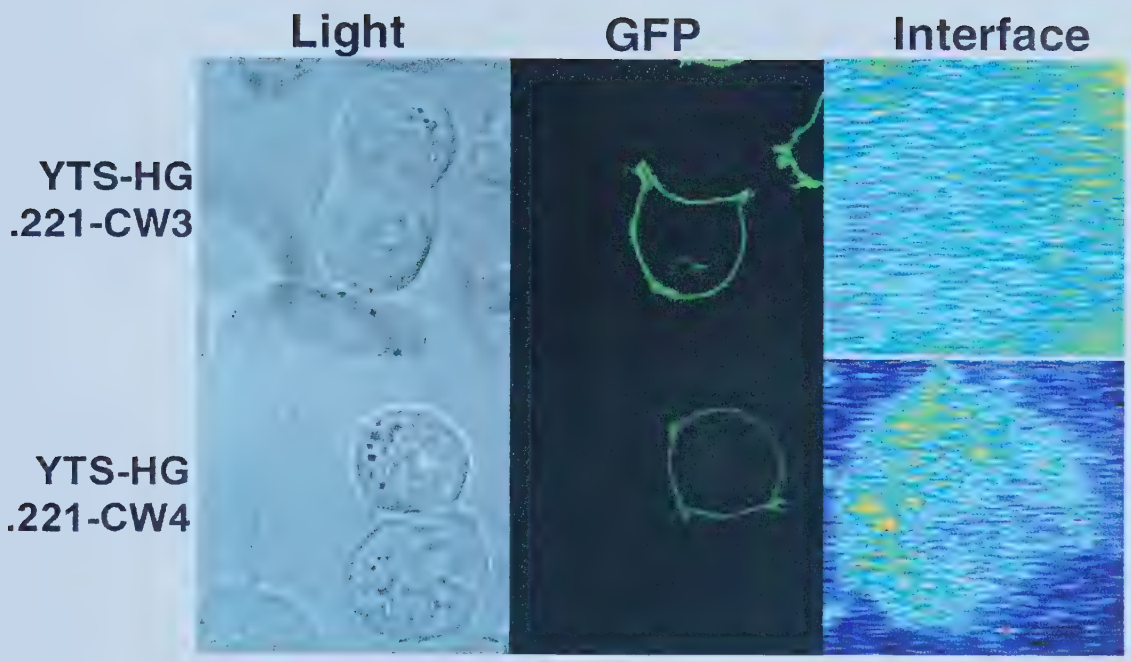


Figure 11. *Ability of 2DL1-HG to cluster with ligand bearing target cells.* YTS-HG cells were combined with unprotected (221-Cw3, top) or protected (221-Cw4, bottom) target cells. Images of conjugates were collected as previously described in figure 10.

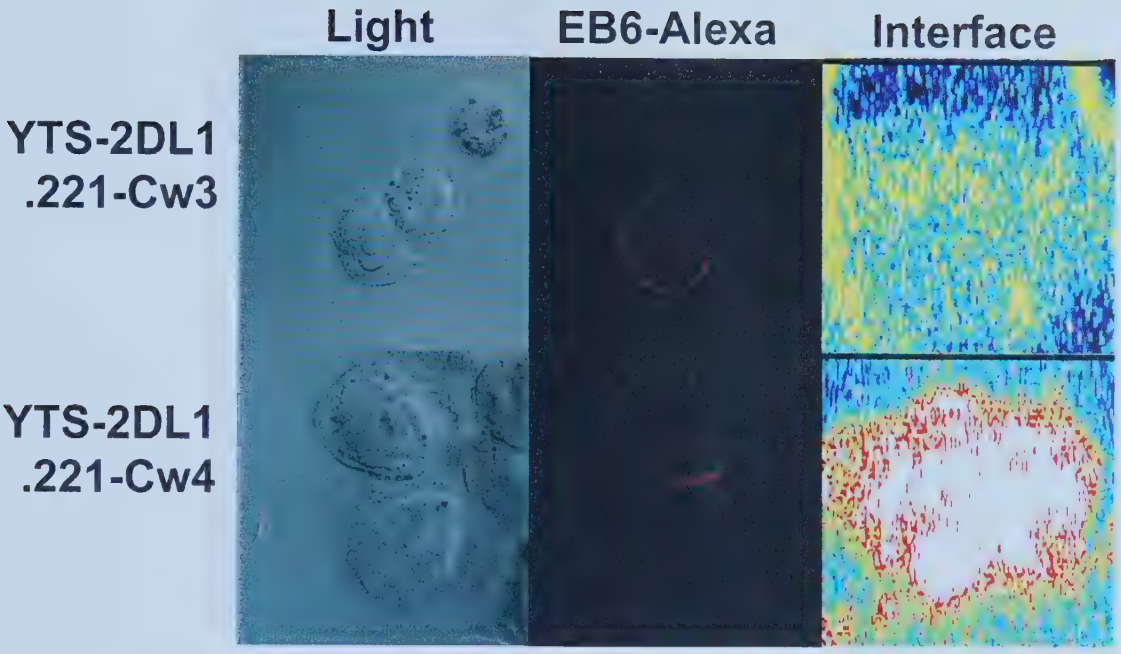


Figure 12. *Clustering of wild type KIR 2DL1 in target cell conjugates.* YTS cells expressing wild type KIR 2DL1 were conjugated with target cells and subsequently fixed and permeabilized. KIR2DL1 was visualized by staining with the EB6 antibody that had been coupled directly to the ALEXA 568 fluorochrome. Z-series of the transmitted light (left panel) and EB6 fluorescence (middle panel) were collected for each conjugate. The interface region was reconstructed as previously described in figure 10.

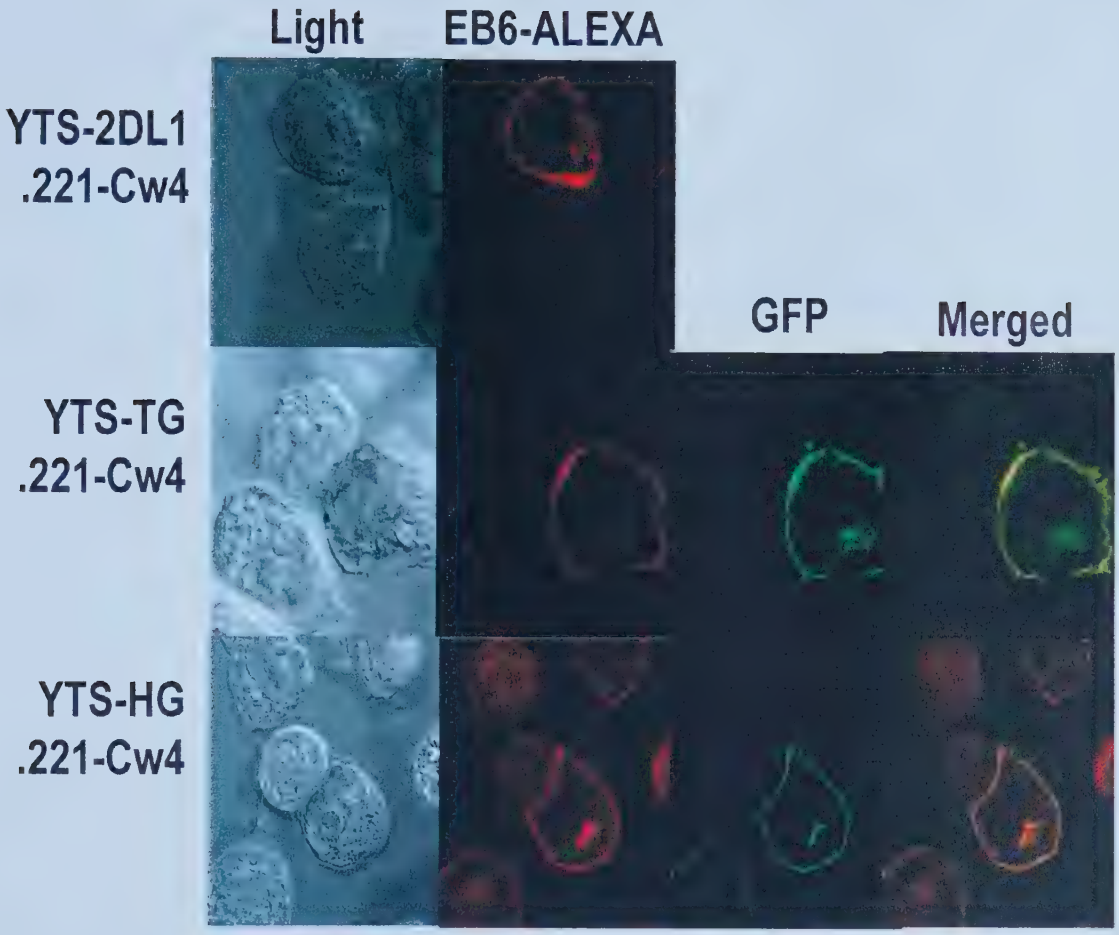


Figure 13. *Correlation of EGFP-KIR clusters with EB6 staining.* Antibody specific for KIR2DL1, EB6, was directly conjugated with fluorescent ALEXA 568. YTS lines expressing either wildtype 2DL1 (top), 2DL1-TG (middle), or 2DL1-HG (bottom) were conjugated with target cells for 20 min at 37°C and subsequently fixed and stained. Conjugates were imaged by confocal microscopy.

Chapter 4. KIR clustering requires activation but not upregulation of adhesion.

4.1 Introduction

Our previous results indicate that macromolecular KIR clustering is not required for the generation of inhibitory signal but it may play an important role in the amplification of signal. KIR clustering may be of greater consequence under conditions of strong activation. Therefore, to understand the mechanisms by which NK cells arrive at self / nonself determinations, a full understanding of the mechanisms by which receptors may modulate the degree of signal is required.

A fundamental question in the field of receptor dynamics is, how do the specific receptors congregate at the target:effecter interface in relatively short period of time? As discussed in Section 1.5, studies in T cells have implicated actin-based strategies that rely on signal complex formation with the cytoskeleton (113). For example, the TCR can attach to the actin cytoskeleton by activation of downstream adapters such as Vav and SLP-76 (124). With the use of inhibitors, myosin motors have been shown to be important in the movement of this receptor into the contact region(112). Models put forward by Dustin and colleagues suggest that TCR phosphorylation may be required for TCR attachment to cytoskeletal components and subsequent movement to the interface(124). However, despite characterized roles in signal transduction and receptor internalization, it remains to be determined if the phosporylation state of the TCR plays a role in the movement of the receptor to the interface.

Similarly, when a natural killer cell engages a potential target cell Src and Syk family kinases become activated and lead to the phosphorylation of KIR ITIM tyrosine residues. Once phosphorylated these residues allow SHP-1 binding and phosphatase activity and which carries out downstream inhibition events. It is unknown if KIR trafficking to the interface functions by any analogous mechanisms to that of the T cell.

The group of experiments presented in this chapter stem from the initial confocal observations with YTS-MG. The functional characterization of this receptor, which has a truncation of the cytoplasmic tail deleting the ITIM motif, is found in Chapter 3 (Figures 4, 5, and 6). As expected, this receptor failed to inhibit both lysis and conjugate formation compared to the wildtype receptor. Initial observations by confocal microscopy seemed to indicate that this receptor was deficient in its ability to cluster. However further observations at later time points indicated that receptor clustering was not abrogated but rather altered. This chapter details a series of experiments originally intended to further describe the behaviour of an ITIM deficient KIR receptor and how they have been extended to novel observations about the behaviour of inhibitory KIR during target cell contact events.

4.2 Cytoplasmic tail deletion alters the ability of KIR to cluster.

If KIR clustering required either association of an adapter protein or receptor phosphorylation, one could predict that a receptor with a deletion in the cytoplasmic domain would exhibit an altered ability to cluster. The 2DL1-MG

receptor contained a truncation of the cytoplasmic tail, deleting the ITIM region with 40 amino acids remaining in the cytoplasmic tail. We combined YTS transduced with the 2DL1-MG receptor with .221 target cells expressing MHC. We imaged conjugates by confocal microscopy and reconstructed the interface region. YTS-MG conjugates clustered receptor at the target:effector interface however, the phenotype of this clustering did not match that seen with YTS-TG (Figure 14). Receptor clustering with YTS-MG was punctate and diffuse compared to a condensed singular cluster of YTS-TG with ligand bearing targets.

The altered KIR clustering pattern of YTS-MG suggested a myriad of possible mechanisms including; inhibitory receptor exclusion from the SMAC, reduced inhibitory cluster stability, or defective inhibitory receptor trafficking. In the first scenario KIR clustering occurs similar to that observed in the full length KIR-EGFP constructs however, SMAC formation or degranulation disrupts the phenotype of KIR clusters. Alternatively, the deletion of the ITIM containing region may destabilize formed KIR clusters, which may be normally stabilized by SHP-1 binding or adapter protein interactions. Thirdly, KIR trafficking to the interface region may be dependent upon the association of KIR, or a KIR associated protein, with cytoskeletal components. We began to explore these possibilities by observing the effect of biochemical inhibitors on KIR clustering to gain some preliminary insight into its' mechanism.

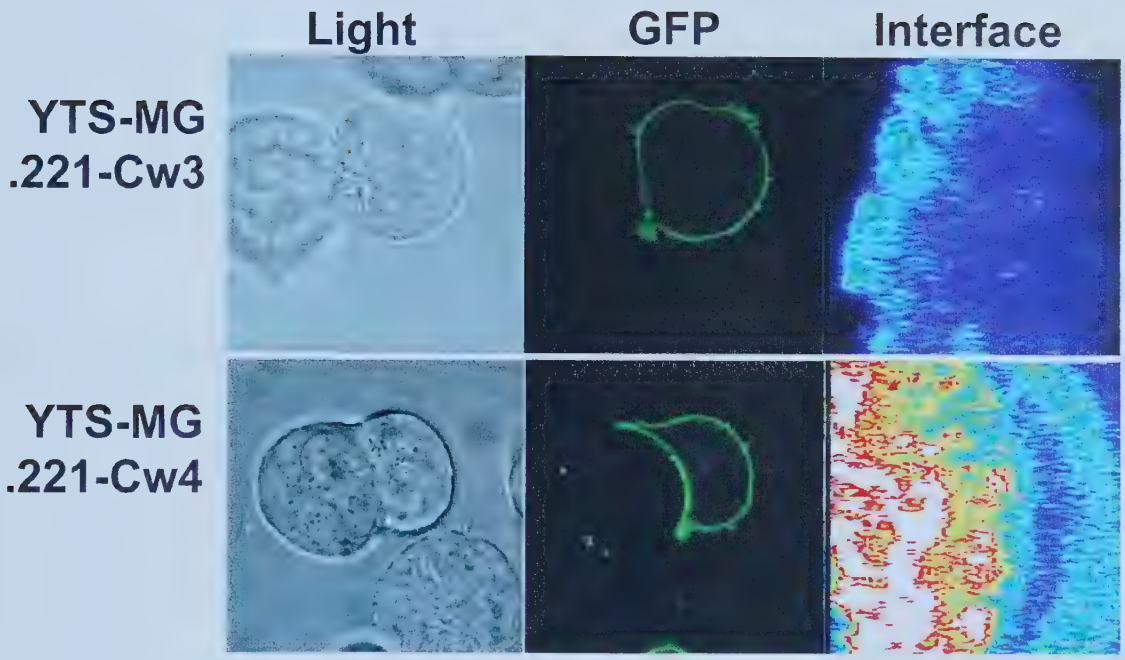


Figure 14. *Ability of the 2DL1-MG chimeric receptor to cluster in YTS.* YTS lines expressing KIR 2DL1-MG were combined with target cells expressing HLA-Cw3 (top) or HLA-Cw4 (bottom) ligand. Live conjugates were imaged by confocal microscopy at 37°C with 5% CO_2. Images were analyzed as previously described in figure 10.

4.3 Biochemical Inhibitor Studies

4.31 Overview of Biochemical Inhibitors

Our system of visualizing receptor clustering in live cells at physiologic conditions lent itself to easy manipulation with biochemical inhibitors. We proceeded to dissect the potential signalling pathways that facilitated KIR clustering with a panel of inhibitors that included PP2, piceatannol, rottlerin, LY294002 and cytochalasin D. I utilized concentrations of inhibitors that have previously been reported specific for their given target (see table I).

Table I. Properties of Biochemical Inhibitors

Drug	Target(s)	IC_{50}	Concentration Used**
PP2	Lck Fyn Hck	4nM 5nM 5nM	10μM(125)
Piceatannol	Syk PKC	10μM 8μM	25μg/mL(126, 127)
Rottlerin	PKC δ PKC α,β,γ PKC ε	6μM 42μM 100μM	100μM(128, 129)
LY249002	PI3 kinase	2μM	50μM(74, 130)
Cytochalasin D	f-actin	n/a*	10μM(131, 132)

* There is no published IC_{50} for cytochalasin D.
** References denote publications that have used these drugs

For some of these drugs, such as the Src family kinase inhibitors, YTS-mediated cytolysis is more sensitive than conjugate formation to drug concentration(84). Therefore, we first used a conjugate assay as a measure of the drug effect on activation induced adhesion (Figure 15). We then proceeded to assay the ability of the drugs to affect KIR clustering (Figure 16, 17, 18), the results of which will be discussed in more detail in the next section.

4.32 Effect of Src and Syk Inhibition on KIR Clustering

The first step in the activation of cytolytic lymphocytes is the phosphorylation and activation of Src family kinases. PP2 is a Src tyrosine kinase inhibitor that effectively blocks both adhesion and cytolysis of NK cells at 10μM (84, 125). We preincubated YTS-TG with PP2 at this concentration and added target cells expressing HLA-Cw4. Compared to the DMSO vector only control, PP2 did not affect KIR clustering (Figure 16, second panel). However, unlike normal NK cells, YTS utilize CD28 as an activation receptor. CD28 can function in a src-independent manner and activate the downstream substrates directly (133). To investigate if Syk was required for KIR clustering, I used piceatannol, a Syk family kinase inhibitor, which may also inhibit some PKC function (126, 127). As with PP2, piceatannol did not affect KIR clustering (Figure 16, third panel). Due to the redundancy between both membrane proximal kinases, we utilized both inhibitors concurrently. Addition of both inhibitors did not affect adhesion to a greater extent than that observed with PP2 alone (Figure 15). However, with both inhibitors present, YTS-TG clustering was severely impaired, being deficient in the majority of conjugates observed (Figure 16, bottom panel). These experiments with Src and Syk family kinase inhibitors put forward two postulates for further examination. First, these data suggest that KIR clustering is dependent

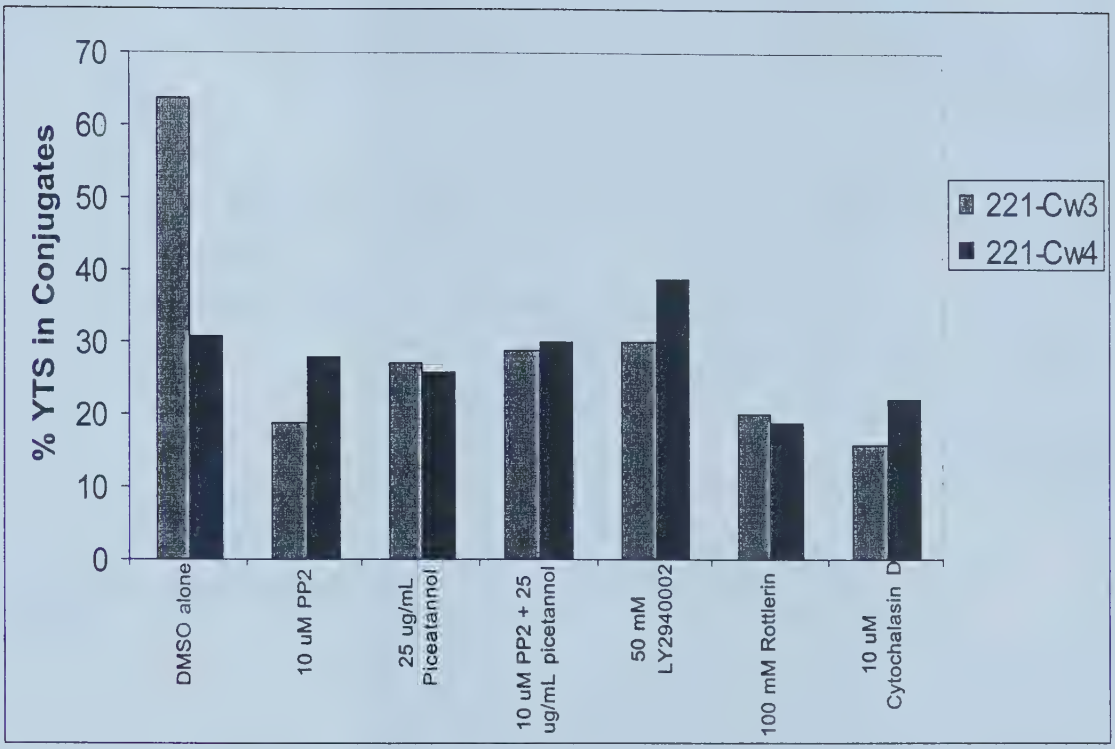


Figure 15. *Effect of Biochemical Inhibitors on YTS Adhesion.* YTS-TG and 221-Cw3 or 221-Cw4 target cells were labeled with red or green membrane dyes respectively (see Materials and Methods). YTS-TG was pretreated with drugs for 30min at 37°C and 5% $C0_2$. Samples were chilled to 4°C, combined, and incubated for 20 minutes at 37°C. Conjugates were read by FACScan as previously described.

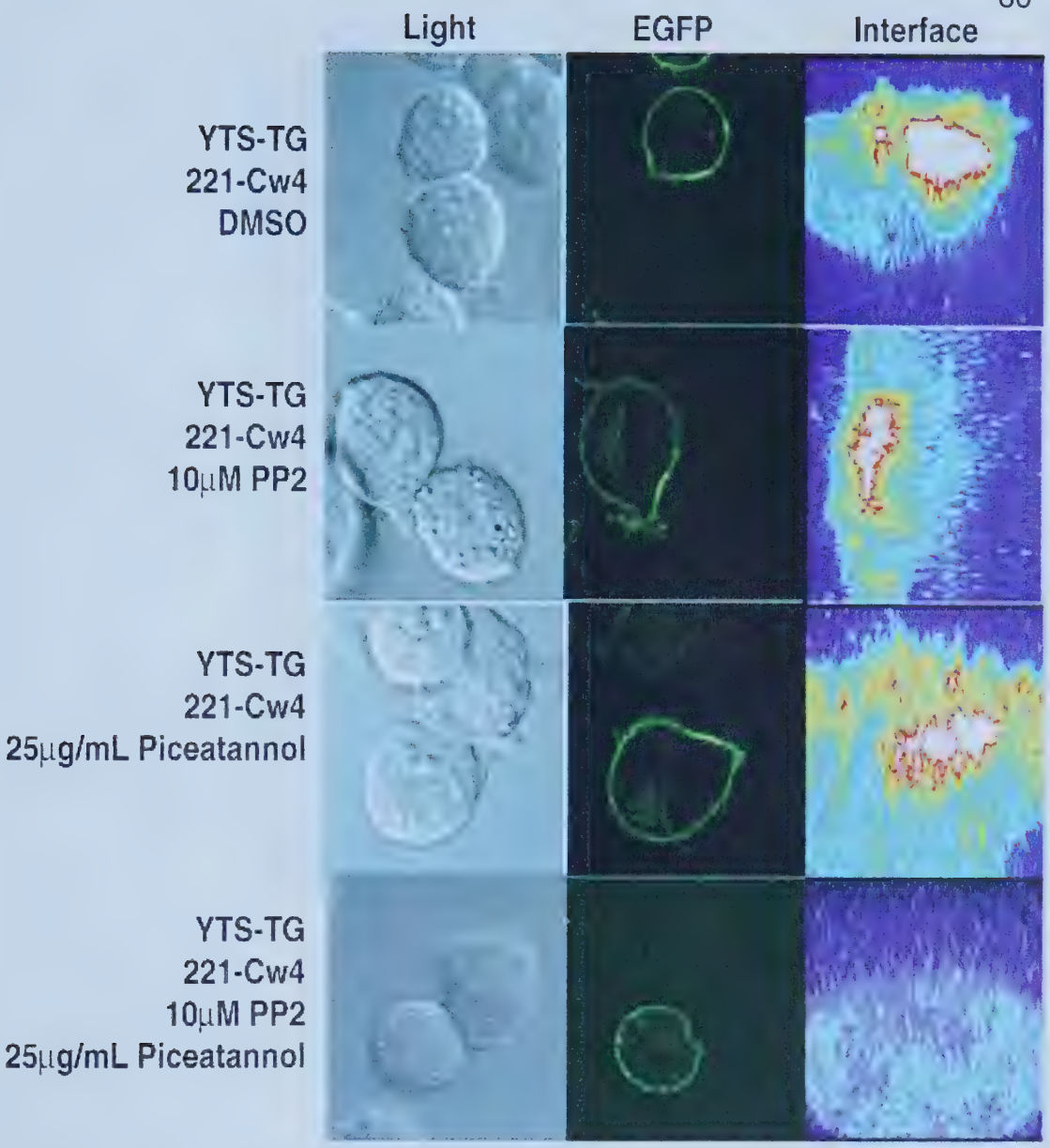


Figure 16. *Effect of the Src and Syk family kinase inhibitors PP2 and piceatannol on KIR clustering.* Live conjugates of YTS-TG with 221-Cw4 targets were treated with either DMSO vector (top), 10μM PP2 (middle top) a Src family kinase inhibitor, or 25μg/mL piceatannol (middle bottom) a Syk family kinase inhibitor, or both inhibitors (bottom).

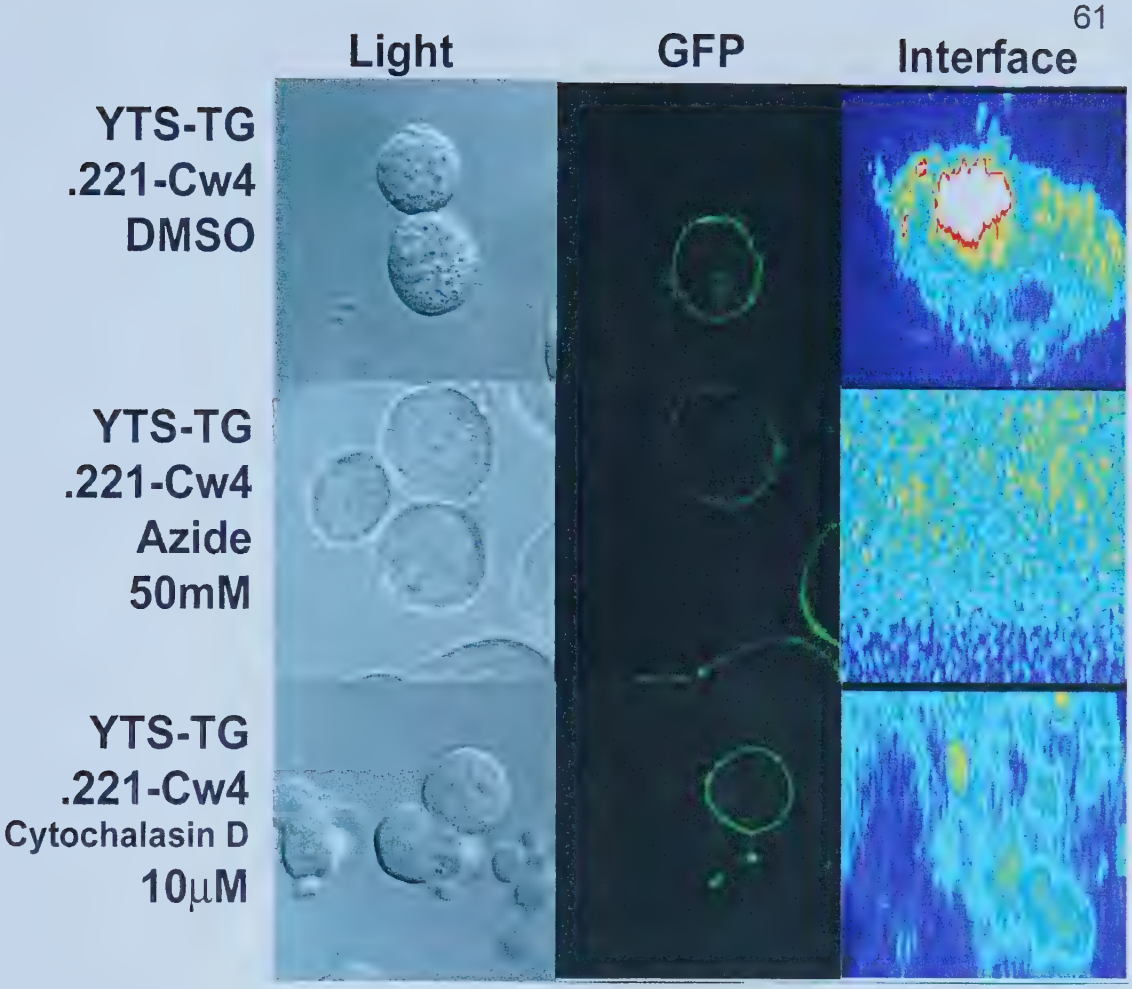


Figure 17. Effect on *KIR clustering of ATP depletion and cytoskeletal disruption.* Live conjugates of YTS-TG with 221-Cw4 targets were treated with either DMSO vector (top), 50mM Azide (middle) an inhibitor of ATP generation, or 10μg/mL cytochalasinD (bottom) a fungal metabolite which disrupts actin polymerization. Conjugates were analyzed live by confocal microscopy.

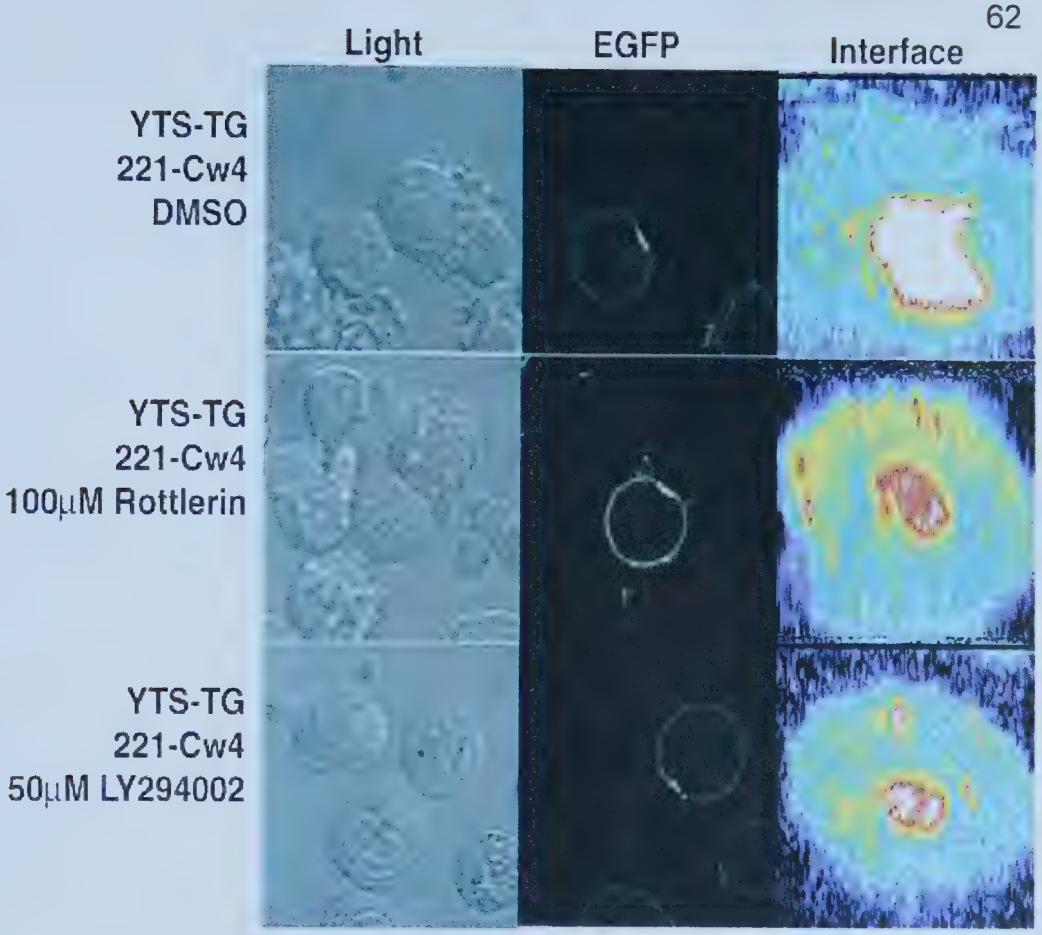


Figure 18. *Effect of PKC and PI3K inhibitors on KIR clustering.* YTS-TG was conjugated with ligand bearing target cells after 30 minute pretreatment with either Rottlerin, a pan specific PKC inhibitor, or LY249002, a reversible PI3K inhibitor. Conjugates were imaged by confocal microscopy.

on cellular activation, specifically the activity of Src, Syk or PKC family kinases. Second, taken together with data showing blockage of conjugate formation (Figure 15) by these inhibitors the above findings suggest that KIR clustering may not require tight adhesion

4.33 Effect of Actin Cytoskeleton Dissolution on KIR Clustering

One of the characteristic hallmarks of lymphocyte activation upon contact with a target cell is actin polymerization, or capping, at the target:effector interface (124, 131, 132). It's also known that, the KIR associated phosphatase SHP-1 can localize with actin cytoskeletal associated components (134); this may allow trafficking of KIR to the target:effector interface. Davies and collegues observed that KIR induced ligand clustering was unaffected by blockage of actin polymerization(91).

In T cells, the accumulation of actin functions to bring antigen receptor to the correct location and provide scaffolding to allow docking of signalling and adapter proteins. Cytochalasin D is a fungal metabolite that blocks the polymerization of the actin, and results in the net depolymerization of intracellular actin and is able to block conjugate formation (Figure 15). In contrast to the findings by Davis *et al.*, in our system cytochalasin D addition blocked KIR clustering (Figure 17). We observed that this effect was reversible: when effector cells were pretreated and the inhibitor subsequently washed out, KIR clustering occured to a similar or greater extent than that observed in the untreated conjugates. The effect of cytochalasin D on KIR clustering may be occurring by two nonexclusive mechanisms. One, KIR may be utilizing the cytoskeleton to

localize to the contact region. Alternatively, KIR activation may be inhibited by dissolution of actin scaffolding that may prevent signal transduction through Src and Syk family kinases.

Active cellular metabolism to supply ATP is required for both the activation of Src and Syk family kinases and actin capping. Therefore we sought to confirm the results outlined above by depleting cellular stores of ATP. Treatment of YTS-TG with the ATP depleter azide completely blocked the ability of KIR to cluster (Figure 17).

4.34 Effect of PKC and PI3K Inhibition on KIR clustering

Other downstream kinases that are also key players in cellular activation include the PKC family of serine theronine kinases and the lipid kinase phosphatidylinositol 3-kinase. Both kinases have been implicated in processes of cytoskeletal remodelling during cell contact events(74).

The protein kinase C (PKC) family has come into importance recently for new discoveries on their subcellular localization and function within T lymphocytes (106, 128, 129, 135, 136). Rottlerin is a panspecific PKC inhibitor that is able to inhibit multiple members of the PKC family including PKC α, β, δ, γ,and θ (137). At a concentration capable of inhibiting all members of the PKC family specified here, rottlerin inhibited conjugation of effector and target cells (Figure 15). KIR clustering, at 100mM concentration of inhibitor, is not affected (Figure 18, middle panel).

LY249002 reversibly inhibits phosphatidylinositol-3-kinase (PI3K), a lipid kinase that has been shown to be important for cytoskeletal remodelling and lymphocyte activation (74, 130). Inhibition of PI3K with LY249002 blocked tight adhesion between YTS-TG and target cells expressing nonprotective KIR ligand (Figure 15). Like Rottlerin, high concentrations of this drug were unable to inhibit or alter KIR clustering (Figure 18, bottom panel). At concentrations of drug sufficient to reduce adhesion, neither PKC nor PI3K inhibitor were able to block KIR clustering.

4.35 Summary of Biochemical Inhibitor Data.

Although the above data fails to fully examine the true nature of the defect exhibited in YTS-MG conjugates, it does postulate two requirements for KIR clustering. First, KIR clustering requires membrane proximal activation signals. Secondly, KIR clustering requires an actin cytoskeleton. Furthermore, the lack of a correlation between KIR clustering and conjugate formation suggests that tight adhesion (controlling conjugate formation) is not required for KIR clustering. This finding is examined in more detail in the following section. The table below summarizes the data presented in section 4.3 (see Table II).

Table II. Summary of Biochemical Inhibitory Data

Inhibitor	Inhibition of..	Effect on Conjugate Formation.	Phenotype of Clustering
PP2	Src	Block	Full clusters
Piceatannol	Syk	Block	Full clusters
PP2 + Piceatannol	Src + Syk	Block	No clusters
Cytochalasin D	f-actin	Block	No clusters
Azide	ATP	N/a	No clusters
LY249002	PI3K	Block	Full clusters
Rottlerin	PKC	Block	Full clusters

4.4 Effect of anti-LFA-1 antibody on KIR clustering.

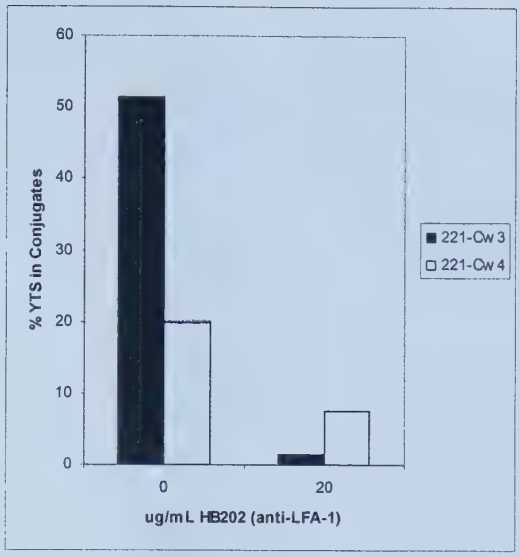
Taken as a whole, the data in figures 15, 16, and 17 suggest that KIR clustering may be independent of tight adhesion between YTS and the target cell. The lack of strong adhesion observed in figure 15 correlates with clustering observed in the presence of the PP2 drug (Figure 16). A criticism of this conclusion is that we may be observing clusters in the proportion of conjugates (30%) that remain tightly adhered to the target cell. The nature of our system, the length of time required imaging a single conjugate, and the frequency of conjugates make collection of a large (n>30) sample of data prohibitive. We therefore employed an alternative approach to test if tight adhesion of YTS to target cells is required for KIR clustering. We used antibody to LFA-1, an activation inducible integrin at the cell surface, to block its interaction with the target cells. Previous studies have found that blockage of LFA-1 prohibits cytolysis and tight adhesion to target cells(82, 84, 138). After preincubating with anti-LFA-1 for 30 min., YTS-TG was combined with HLA-Cw4 expressing targets and analysed by confocal microscopy. Blockage of LFA-1 did not impair the ability of KIR to cluster when blocked with HB202 (anti-LFA-1 antibody) at a concentration of 0.02 mg/mL (Figure 19). We can conclude from this series of experiments that LFA-1 induced adhesion is not required for KIR clustering.

4.5 KIR clustering and the cytoskeleton

4.51 Effect of jasplakinoide on KIR clustering

The abrogation of clustering by cytochalasin D suggested two possible explanations. KIR clustering may require actin-based motility to allow accumulation in the contact region. Alternatively, actin polymers may provide a necessary scaffold upon which activation kinase complexes form to activate cytolysis. To help distinguish between a requirement for actin-based motility (ie; actin polymerization) and a requirement for actin scaffolding (for activation signalling) we treated the cells with jasplakinoide. Jasplakinoide is an irreversible inhibitior of f-actin depolymerization, locking the actin polymers in a filamentous form (139). Therefore despite maintenance of suitable actin sites for the formation of signalling complexes, treatment with this drug would prevent actin turnover and actin based motility processes. When jasplakinoide was added to the target cells alone, no effect on KIR clustering was observed ruling out requirements for actin polymerization in the target cells (Figure 20, third panel). When effector cells were pretreated with jasplakinoide, KIR clustering was not completely inhibited and formed punctate clusters (Figure 20, second panel). This is a phenotype similar to that observed with the 2DL1-MG ITIM deletion KIR receptor. These results suggest that a dynamic actin cytoskeleton is required for the facilitation of wild type KIR clusters. However, the possibility that jasplakinoide may be simply blocking attachment of adaptor proteins to the cytoskeleton and preventing activation cannot be ruled out.

a.



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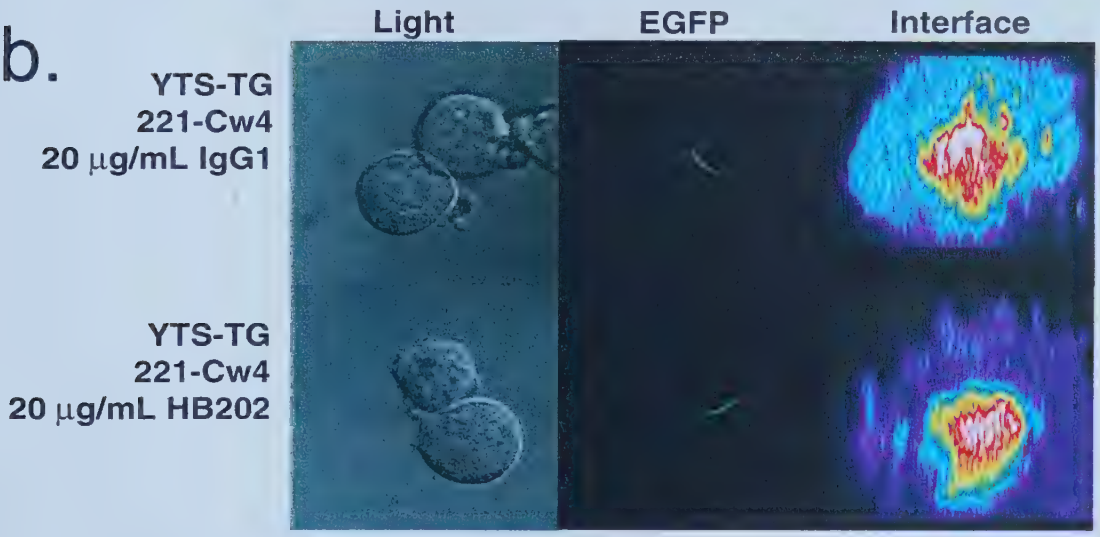

Figure 19. *Effect of anti-LFA-1 antibody on KIR clustering.* a) YTS-TG and target cells expressing either HLA-Cw3 or HLA-Cw4 were labeled with green or red membrane dyes. Cells were incubated with 20 μg/mL of HB202 or CWY-3 (isotype control) antibody for 30 minutes at 4°C. Effectors and targets were combined and warmed to 37°C for 20 minutes. Samples were then vortexed and immediately fixed and analyzed for conjugates by FACScan. b) Cells were preincubated with antibody for 30minutes. Following incubation YTS-TG was combined with targets expressing HLA-Cw4 and imaged as previously described in figure 8. Anti-LFA-1 antibody (HB202) and isotype control antibody (CWY-3) was maintained at 20 μg/mL throughout the experiment.

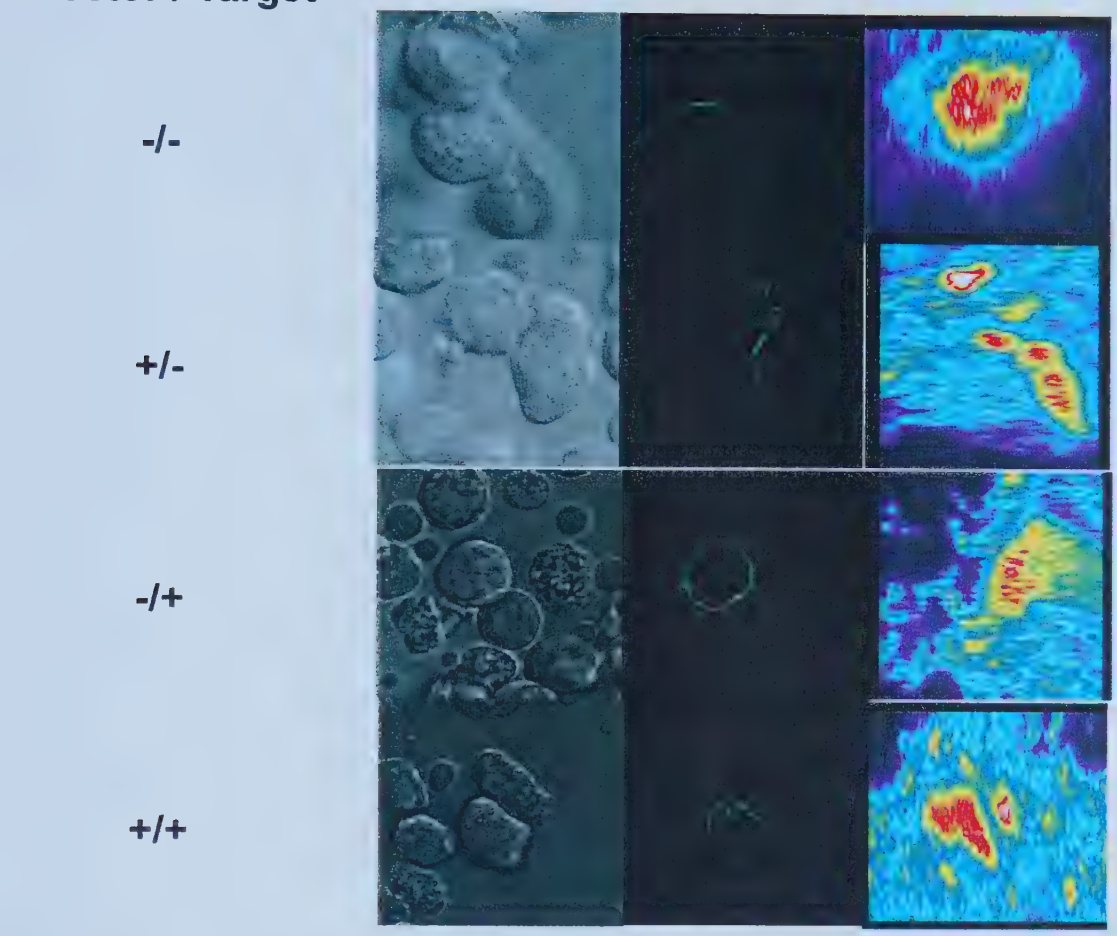

Figure 20. *Blockage of actin depolymerization mildly effects KIR clustering.* YTS-TG conjugates were imaged live after pretreatment of either the effector and/or the target cells with 10μM Jasplakinoide, a small molecule inhibitor of the actin depolymerization. Conjugates were imaged by live cell confocal microscopy.

4.52 KIR clustering locally excludes actin polymerization

We have found that cytoskeletal inhibitors affect the ability of KIR to cluster. Our data suggests a functional link between KIR clustering and the actin cytoskeleton. We initially hypothesized that KIR and actin should colocalize in inhibitory conjugates, the rationale being that KIR trafficking to the contact zone required new f-actin formation. One of the caveats of visualizing the effector actin cytoskeleton in a fixed conjugate was the contribution of the target cell actin cytoskeleton to the interface. In addition to irreversibly binding f-actin, jasplakinoide blocks the binding of phalloidin, a fungal metabolite which reversibly binds f-actin. Therefore we masked the contribution of the target cytoskeleton by pretreatment of the target cells with jasplakinoide. As previously shown, treatment of target cells with jasplakinoide does not prevent KIR clustering (Figure 20). We conjugated YTS-TG with both protected and unprotected targets for a time course. These conjugates were subsequently fixed and stained with ALEXA568-phalloidin and visualized under confocal microscopy. In an unconjugated state, no clustering of either polymerized actin or KIR was observed (Figure 21, top panel). When YTS-TG conjugates with 221-Cw3 targets were observed, actin and KIR accumulations localized together in the reconstructed interface (Figure 21, left panel). When conjugated with protective ligand bearing targets KIR, as shown previously, clustered as expected (Figure 21, right panel). The localization of actin at the site of an inhibitory KIR cluster was reduced in the region occupied by the central cluster of KIR. In accordance with our data, ILT, a receptor that is able to activate SHP-1 through

an ITIM region similar to KIR can block actin polymerization in T cells stimulated by anti-TCR antibody-coated beads (140).

Our results, described above, showing KIR clusters segregated from actin polymerization, suggested two possible explanations. First, clustered KIR receptors may simply be physically excluding activating receptors from accessing the central interface region, preventing formation of a SMAC and putatively, a competent activation signal. Alternatively, KIR signalling events, mediated through a functional ITIM domain, may be facilitating the exclusion process. To determine if the exclusion of actin staining from the central core of clustered KIR was a result of signalling or simply a result of activation receptor exclusion we compared the ability of YTS-MG to exclude actin to the full length receptor construct. YTS-MG, as previously described, is able to cluster in disorganized, punctate structures. However, due to a deletion in the ITIM region it is unable to inhibition cytolysis or adhesion when conjugated with a target cell expressing protective ligand. Performing a similar experiment to what has been previously described above we incubated jasplakinoide pretreated targets with YTS cells expressing either the 2DL1-MG or 2DL1-TG receptor. Following incubation, samples were fixed and stained with ALEXA568-phalloidin. Conjugates were imaged by confocal microscopy. We found that the YTS-MG receptor was unable to reduce the accumulation of actin in the region where KIR accumulated (Figure 22). This suggests that actin exclusion from KIR clusters is mediated by SHP-1 activation or by another as yet uncharacterized process mediated by the KIR cytoplasmic ITIM region.

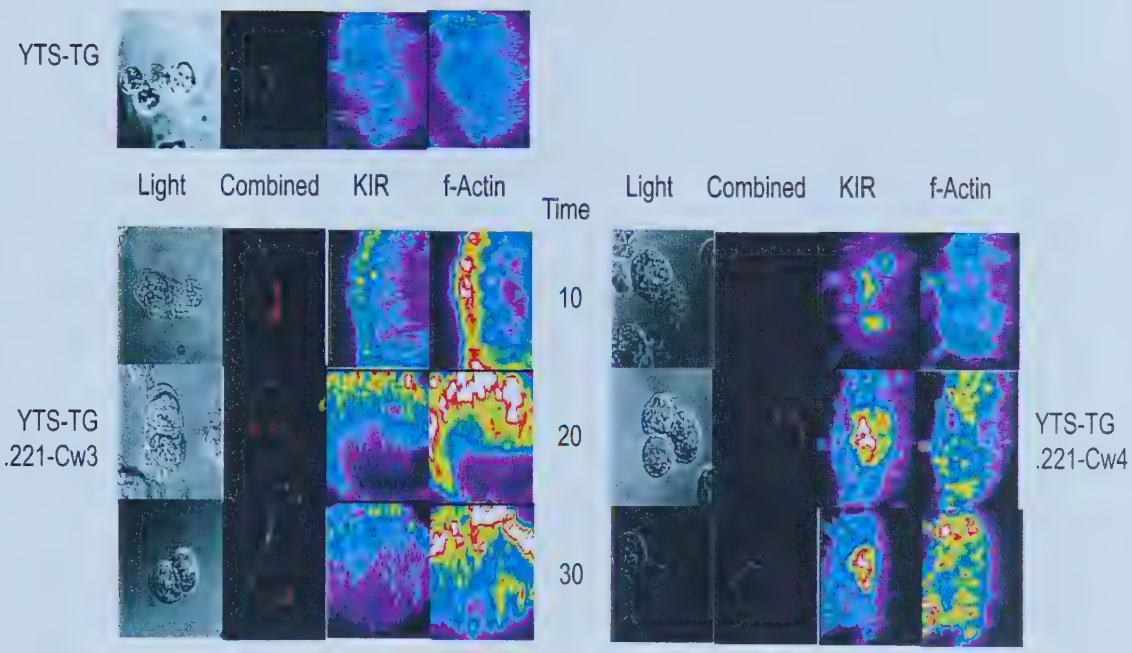

Figure 21. *Costaining of Actin and KIR at an Interface*. To mask phalloidin staining, target cells were pretreated with 10μM jasplakinoide for 30min at 37°C. Conjugates of YTS-TG with 221 target cells were fixed and permeabilized after mixing and co-incubation for the indicated time course. The samples were then stained with ALEXA 568 conjugated phalloidin. Conjugates were imaged by confocal microscopy.

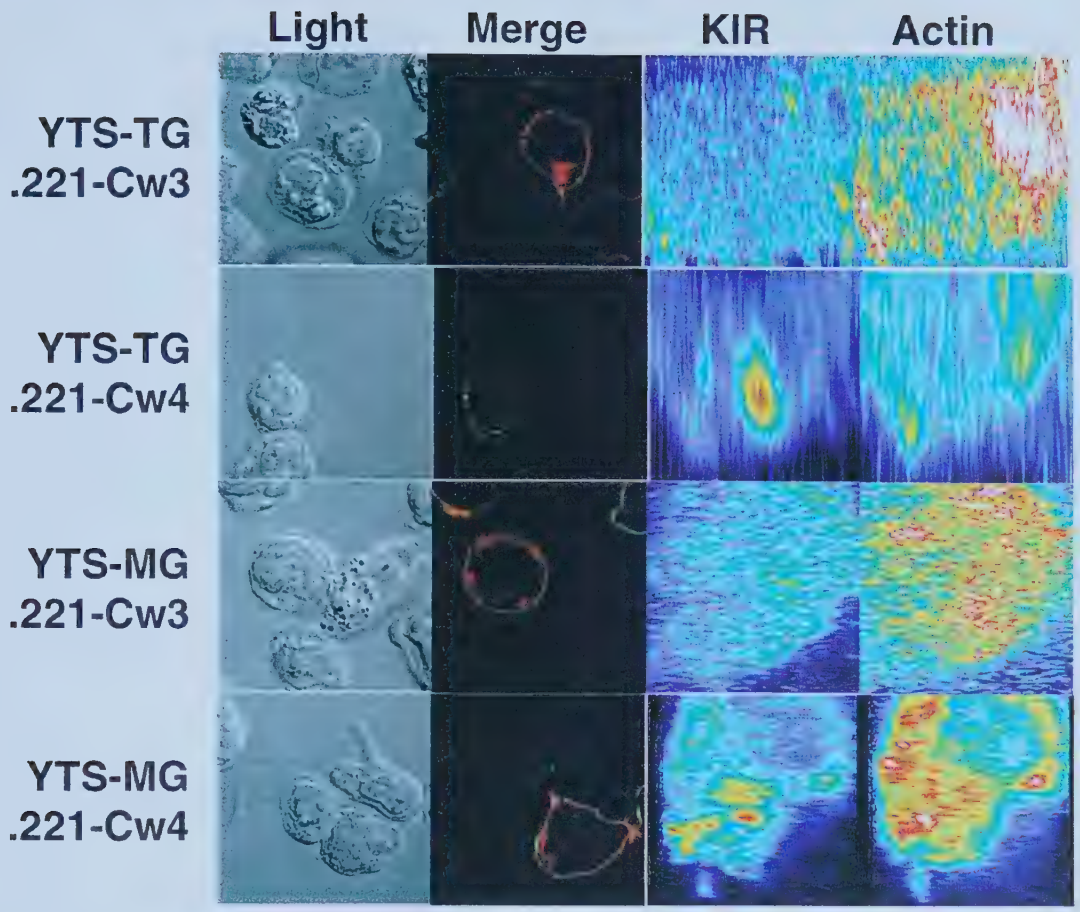

Figure 22. *Cartography of Actin and KIR at the Interface*. YTS conjugates were preincubated with target cells for 20min. Sampled were prepared and imaged as in figure 19.

4.6 Summary of Results

The results presented in this chapter detail a series of experiments that directly conflict with a few of the findings of Davis *et al.* (91). The explanations for these differences may be technical including the temperature of analysis for the live conjugates, or Davis' use of Ab staining which may block the function of adhesion receptors with live cells. In addition to these technical issues, the differences may simply be kinetic; perhaps observing KIR clusters coalescing or disassembling.

KIR clustering represents a valuable function of KIR, providing a means to amplify signal in situations of low levels of surface receptor. Above we have outlined some mechanisms by which KIR clustering is promoted. We show that the cytoplasmic tail deletion alters the ability of KIR to cluster. Despite a continued ability to cluster, the phenotype observed is a punctate pattern that is markedly different from wildtype. The reduction of conjugate formation with the use of drug inhibitors or blockage of LFA-1 with antibody does not affect KIR clustering. We therefore postulate that LFA-1 mediated adhesion, or conjugate formation, is not required for KIR clustering. However, upstream activation signalling may be important to facilitate the generation of KIR clustering. In contrast to the results reported by a previous group (91) we observe that KIR clustering is dependent upon the dynamic nature of the actin cytoskeleton. Finally, we demonstrate that KIR clustering decreases the intensity of actin staining, and that this is dependant upon a full cytoplasmic domain containing an ITIM.

Chapter 5. Conclusions and Future Directions

5.1 Summary of Findings

Today, we know that NK cells utilize a vast array of activating and inhibitory receptors pairs to coordinate cytotoxicity however, the overlying questions remains, how does an NK cell distinguish friend from foe? We hypothesized that the ability of KIR to cluster and amplify the inhibitory signal when engaged on ligand, plays a key role in cytolysis. To this end we investigated if inhibitory signalling required KIR clustering as well as the processes controlling KIR clustering itself.

In T cells the generation of appropriate activation signalling is coincident upon the formation of a mature SMAC or immune synapse (107). Accordingly in the epidermal growth factor receptor system, inhibition of receptor clustering can alter or block signal generation (105). Furthermore, inhibitory receptor co-clustering is required to block activation signalling and to generate inhibitory signals (64, 103). These findings support a paradigm wherein receptor clustering leads to signal generation.

The first study that investigated the phenotype of KIR clustering was published by Davis *et al.* (91). I have previously discussed their work in section 1.6. In review, Davis *et al.* tagged MHC with EGFP, transfected into target cells, and stained ICAM-1 to determine the position of KIR and LFA-1 receptors in conjugates. They showed that KIR clustered at the interface between YTS (an NK cell line) and targets, describing the clustering as part of an inhibitory immune synapse in which KIR clustered around a central core of LFA-1 adhesion

receptors. Moreover, testing a panel of diverse inhibitors, such as azide and cytochalasin D, they found that only zinc chelation affected the ability of KIR to cluster. This adds to the previous findings where zinc binding was shown to be vital for KIR functions, including signal transduction and dimerization (57, 58, 102), and suggested a predicted role for zinc in KIR clustering.

Some of the findings of Davis *et al.* may conflict with previous reports on the mechanism of KIR function if KIR clustering is coincident with inhibitory activity. Studies have indicated that KIR function is dependent upon cellular activation (ie; activity of Src and Syk family kinase) to phosphorylate the ITIM (63, 141). Though it is not known how these kinases mediate the phosphorylation of KIR, it is thought that functioning of these kinases is dependant upon a large signalling complex that requires attachment to cytoskeletal components. Furthermore, phosphorylation, and by inference a cellular source of ATP, is required for ITAM recruitment of these kinases and their activation. If KIR receptor fails to function without co-clustering of activation receptors, is KIR clustering independent of activation signalling? If clustering is not an active cellular process, how does KIR receptor congregate at the interface within three minutes as reported by Davis *et al.*? With these apparent paradoxes in mind, we attempted to determine more about the biological implications of KIR clustering and the mechanisms controlling it.

5.2 KIR signalling is not dependent on KIR clustering

We generated KIR-EGFP chimeras and set about analyzing their ability to cluster in our system. We utilized functional assays and compared those data to our findings regarding receptor clustering by confocal microscopy. Our data is summarized below:

1.) KIR signalling is independent of large macromolecular KIR clustering. We show in figures 5 and 6 that both the KIR-EGFP chimeras are able to inhibit YTS cytolysis and adhesion to target cells. Taken togther with figures 10 and 11, we show that despite receptor function in the YTS-HG cell lines, macromolecular KIR clustering is not detected. This data is significant in that it is the first example of inhibitory receptor function in the absence of visible receptor clustering.

2.) KIR clustering enhances KIR signalling. If KIR clustering is not required for KIR signalling, what type of advantage does KIR clustering allow? To answer this question we titrated the level of receptor expression and assayed for the ability to inhibit NK92 cytolysis (Figure 8). We find that at a low receptor level 2DL1-HG (N-terminal EGFP-KIR) is unable to mediate the same degree of inhibition as 2DL1-TG (C-terminal EGFP-KIR).

3.) KIR clustering is not solely dependent on the zinc binding motif. The defect in the 2DL1-HG receptor may be easily explained if the receptor had altered its requirement for zinc to potentiate an

inhibitory signal. We blocked the inhibitory function of both 2DL1-TG and 2DL1-HG with a zinc chelator (Figure 9). These data indicate that zinc binding is functional in the 2DL1-HG receptor.

These findings, and those of the previous group (91), have suggested a two-step model for the clustering of KIR upon ligand engagement (see figure 23). KIR signalling is controlled by zinc-mediated dimerization or lower order multimer formation. This structure is dependent upon zinc to function and is capable of transducing a competent inhibitory signal in NK92 at receptor densities comparable to that of primary NK cells. This accounts for the observations by Davies *et al.* of a blockage of KIR clustering with the addition of zinc chelator. We have confirmed this initial step by noting the sensitivity of both the 2DL1-TG and 2DL1-HG receptors to zinc chelation, and the ability of both receptors to transduce an inhibitory signal. Receptor clustering then proceeds to form large, visible, higher order multimers. We propose that formation of this multimer is physically blocked by the addition of the EGFP moiety to the N-terminus of KIR2DL1. EGFP blocks a putative interaction between KIR dimers and prevents lattice formation and visible receptor clustering formation.

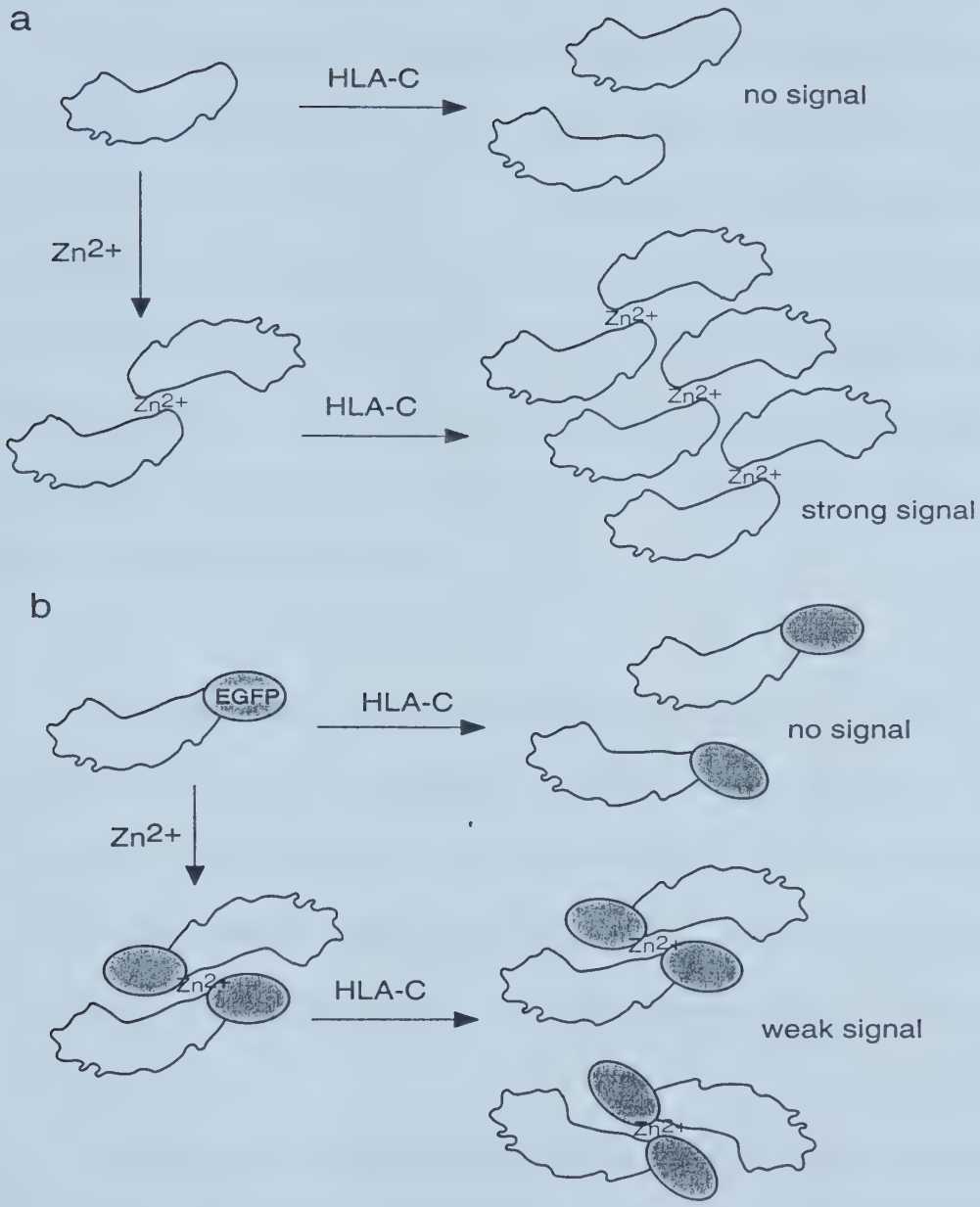

Figure 23. *A two step model for KIR clustering.* Reproduced with permission from E.O. Long. Portrayed in the top down view. A) In the normal circumstance, KIR signaling is initially controlled by dimer formation, which is facilitated through interactions at the zinc binding motif. Further multimerization is due to interactions of a second domain of KIR with HLA-C to form a multimeric lattice. B) Representative of the behavior of 2DL1-HG, where the EGFP moiety is putatively interfering with multimerization but not signal generation.

5.3 KIR clustering is independent of LFA-1 adhesion

As stated previously, inhibitor studies done in this paper conflict with the current model of KIR function (91). To address some of the questions remaining after the initial work by Davis *et al.* we had attempted to discover more about the mechanisms surrounding KIR clustering. Again, we used confocal microscopy of YTS-TG conjugates with target cells and treated these conjugates with either a panel of biochemical inhibitors, or blocked receptor function with antibodies. This body of work yielded three distinct findings on the nature of KIR clustering in NK cells. These are summarized below:

1.) The ITIM region may play a role in the enhancement of KIR clustering, but is not required for clusters to form. This is shown in clustering data with YTS-MG (ITIM deleted KIR-EGFP) (Figure 14). YTS-MG fails to form a single organized cluster and is disorganized and punctate compared to YTS-TG (full length KIR2DL1 with EGFP at C-terminus) (Figure 10).

2.) We demonstrate that tight adhesion is not required for KIR clustering. Shown in figures 15, 16, and 19 these data show, by two independent methods, that integrin mediated tight adhesion or conjugate formation is not required for inhibitor clustering. First, at drug concentrations that greatly reduce conjugate formation, KIR clustering is unaffected (Figures 15, 16 and 18). Additionally, blockage of LFA-1, the key integrin that facilitates conjugate formation, does not block KIR clustering (Figure 19).

3.) Early membrane proximal activation signals (ie; src and syk family kinases) are required for KIR clustering. We observed that blockade of activation signals results in a blockage of KIR clustering. Though inhibition of either Src or Syk family kinases, with either PP2 or piceatannol alone is not sufficient, utilization of both inhibitors blocks KIR clustering (Figure 16).

Although these data provide evidence for some of the requirements of KIR clustering, they fail to discriminate between three postulates to explain the clustering pattern of YTS-MG conjugates. Exclusion of KIR from the SMAC, reduced inhibitory receptor stability, and defective receptor trafficking, all remain to be tested. To test the possibility that 2DL1-MG is excluding from the SMAC staining of activation receptors (ie; CD28) is needed. Elucidation of reduced inhibitory receptor stability may be accomplished with fluorescence recovery after photobleaching experiments to determine if the kinetics of KIR in a YTS-MG cluster differ from that of YTS-TG cluster. Finally, defective receptor trafficking may be testing by observing the movement rate of KIR ligand coated beads toward the target interface. Clear support for any mechanism of KIR clustering will require high speed, real time imaging of the process of KIR clustering for both full length and ITIM deleted receptors. With further information on the event kinetics, signal protein deficient cell lines (ie; Vav, SLP-76) can be used to delineate individual events.

As with the TCR, movement of a majority of KIR to the target interface by diffusion alone would be predicted to require more than 30min. of contact. It is therefore likely that some active cellular process traffics KIR to the target interface. Further research in this field will be required to discriminate between two broad mechanisms of KIR receptor trafficking: direct attachment or bulk flow. The direct attachment model states that KIR trafficking is facilitated by the attachment of KIR or KIR-associated proteins to the cytoskeletal machinery. This model is supported by findings that detail the association of SHP-1 with vav and actin (96, 142, 143). However, KIR clustering due to this mechanism is unlikely, as the association between these proteins maybe very transient as a result of the SHP-1 catalytic activity. The bulk flow model is a more plausible hypothesis to explain the movement of KIR to the contact region. In this model, KIR receptors are "pulled" along with the activation receptors to the site of contact, possibly via distinct membrane microdomains or by SHP-1 coassociation with other adapters, such as LAT or SLP-76, that have associated with cytoskeleton. Once in the interface region, KIR would become engaged on available ligand, cluster, and proceed to inhibit activation signalling and cytolytic function.

5.4 KIR Clustering and the immune synapse

The dissolution of the T cell immune synapse is controlled by a number of factors including chemokine gradients, receptor density and inhibitory receptor signalling (114, 115). A potential model is that inhibitory receptor engagement, in general, can dissolve or prevent the formation of a SMAC. One of the methods

by which KIR could be affecting SMAC formation is by altering actin polymerization. Some evidence that supports this hypothesis includes: treatment of T cell conjugates with cytochalasin D, an inhibitor of f-actin polymerization, prevents the formation of the SMAC (124) (88) and, Vav activity is necessary for TCR translocation to the target cell interface (143). If SHP-1 is able to catalyze the depolymerization of f-actin polymers, either directly or through effects on Vav, then KIR activity may control SMAC formation. Vav colocalization, phosphorylation state, and exchange activities during NK conjugation may all play roles in determination of the ultimate signalling outcome.

Actin polymerization and capping is a step in the formation of the activating immune synapse. We observed that KIR clustering is segregated from increased actin polymerization (figure 21). The exclusion of actin from KIR clusters is likely dependant upon the activity of SHP-1, shown in figure 22, as YTS-MG fails to segregate from actin. This represents a potential mechanism by which KIR clustering may affect the formation of the activating immune synapse. Putatively, dissolution of the actin cap mediated by KIR, in a mechanism similar to cytochalasin D treatment prevents formation of, or disassembles, the SMAC.

Two mutually exclusive models can be envisaged to explain how KIR clustering might be locally excluded from actin polymerization. In the "upstream action" scenario, active SHP-1 binding to and/or dephosphorylation of Vav blocks the function of this adapter protein to activate actin polymerization. Alternatively, in the "downstream action" scenario KIR activation of SHP-1 phosphatase activity may shift the equilibrium to favor g-actin monomers.

5.5 Future Directions

We have shown here that KIR clustering leads to increased inhibitory signalling. It remains to be shown if KIR clustering is capable of amplifying a signal, or if the increased inhibition is due solely to increases in engaged receptor density. It may also be of interest to determine the crystal structure of the KIR cytoplasmic tail bound to SHP-1, which may reveal novel interactions which allow for amplification of inhibitory signal. Moreover, the role of receptor clustering may be more relevant in an *in vivo* system. KIR chimeric mice have been generated which express KIR and the appropriate MHC ligand. In this type of system, how would the effect of a nonclustering receptor (ie; 2DL1-HG) affect the outcome of receptor expression during NK cell maturation?

One of the most puzzling results in this thesis is the altered, but not defective clustering by the 2DL1-MG receptor. The phenotype of this receptor suggests that KIR phosphorylation, direct attachment to cytoskeleton, or binding of an adapter protein may all faciltate the synergistic movement of KIR in the interface. Real time high speed imaging is required to accurately observe the kinetics of this receptor as compared to its full length counterpart. Understanding the altered behaviour of 2DL1-MG may uncover novel mechanisms of KIR trafficking and regulation.

Stemming from our observations regarding the interaction between KIR and actin at the target cell interface, one of the most intriguing future directions of this work is the potential interplay between clusters of inhibitory and activating

receptors at the interface. Variable engagement of activation and inhibitory receptors in other systems (ie; B cells) allows fine-tuning of the cellular response to the type of antigenic stimulus. The signal may be amplified, modified, or abrogated, depending on the combination of receptors engaged. Moreover, the ability of receptors to increase their local membrane density, or cluster, at the interface of a target cell will play a key role in signal outcome. Engagement of both activation and inhibitory receptors often results in inhibition of activation signalling (54). This finding is predicted by the missing self hypothesis, wherein it is the absence of the inhibitory signal which distinguishes a target cell from an innocent bystander. An important caveat of many of these studies is that most have involved crosslinking inhibitory and activation receptors with antibodies, which may override the effects of the receptor affinities on intracellular signalling during cell-cell contact events. Furthermore, despite the large body of work on the characterization of NK cell receptors, further work needs to be done on their ligand specificity and the coordinate development of antibody reagents capable of distinguishing activating and inhibitory isoforms. This will help identify the role that each receptor plays as activator, costimulator or coreceptor. With these accomplishments in hand, we can start to assess the interplay between these competing signals on the cell surface to determine how NK cells recognise friend from foe.

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Appendix

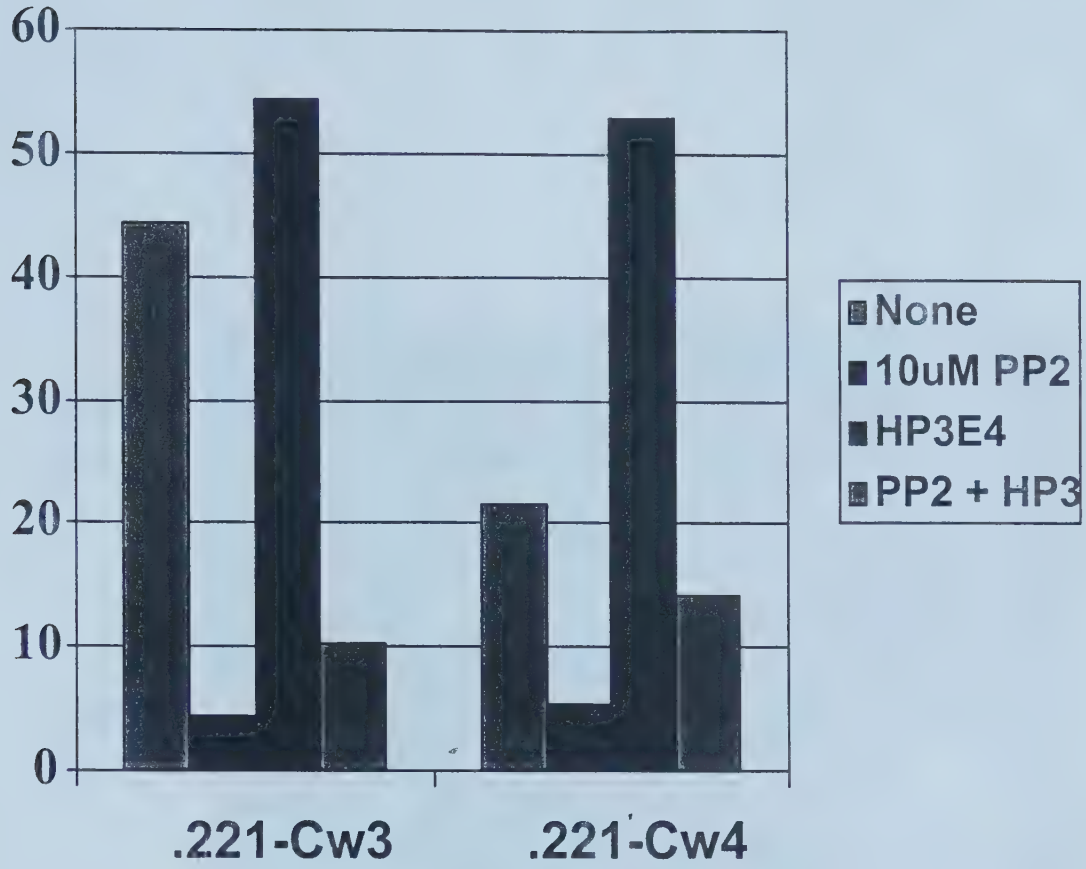

Figure 24. *Conjugate formation of NK92-TG with 721.221 targets*. An NK92 cell line that had been stably electroporated with KIR2DL1-TG was labeled with PKH67 membrane dye and the target cells were labeled with PKH26 membrane dyes. These cells were treated with either DMSO vector alone (green bars), the Src tyrosine kinase inhibitor PP2 (blue bars), IgM antibody specific for KIR2DL1, HP3E4 (red bars), or both HP3E4 and PP2 (orange bars). Targets and effector cells were combined with target cells at 4°C and then incubated for 30 minutes at 37°C. Results were obtained by FACS as in figure 6.

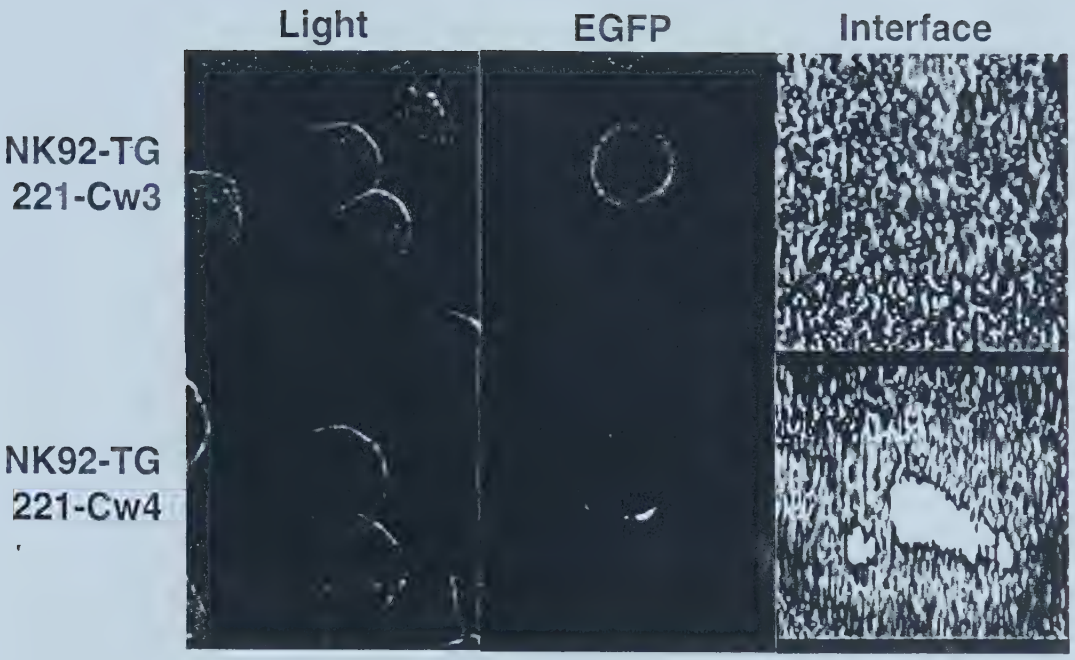

Figure 25. *KIR2DL1-TG stably transfected into NK92 clusters with ligand.*NK92-TG was mixed with target cells expressing either HLA-Cw4 or HLA-Cw3. The cells were centrifuged to induce conjugation and immediately analyzed at 37°C with 5% CO_2 by confocal microscopy.